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TITLE: FAK Signaling in the Acquisition of a Cancerous Phenotype in Breast Epithelial Cells

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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

As a substrate and binding partner for the Src oncogene, a role for the focal adhesion kinase (FAK) in cancer has been speculated. FAK mediates cellular processes including cell motility, survival, and proliferation. Thus, aberrant FAK signaling could lead to pathological effects, including oncogenesis. FAK is overexpressed in a variety of tumors. We have established a cancer cell model system to study the role of aberrant FAK signaling in human cancer. Increased FAK signaling was achieved with the expression of a constitutive activated mutant of FAK, SuperFAK. In contrast, FAK signaling was inhibited using FRNK, a FAK dominant negative. The effect of altered FAK signaling in the acquisition or loss of transformation phenotypes in normal and cancer breast epithelial cells was monitored. Decreased FAK signaling by FAK led to a loss in adhesion independent growth of the breast cancer cells. FRNK had no effect on the proliferation nor the survival of the cells. Elevation of FAK signals by overexpression of the hyperactive FAK mutant increased the tumorigenic capacity of the breast cancer epithelial cells. These data demonstrate the role for aberrant FAK signaling as a cause of some of the phenotypic changes that occur when a cell becomes oncogenically transformed.

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Table of Contents

INTRODUCTION

Focal Adhesion Kinase (FAK) is a non-receptor tyrosine kinase that localizes to focal adhesions, sites of close contact between cells and the extracellular matrix substrate on which they attach (1). The engagement of the integrins by their extracellular matrix ligands causes the integrins to cluster at focal adhesions (2). The treatment of cells by soluble factors and integrin clustering upon cell adhesion activate FAK (3). FAK can autophosphorylate creating a binding site for SH2-containing proteins, including Src (4-8). Once bound to FAK, Src can further phosphorylate FAK leading to full activation of the kinase and creating new binding sites for additional SH2 proteins (6;8-10). In addition, FAK also contains proline rich regions in the carboxy and amino domain which allows for recruitment of SH3-containing proteins, including p130cas (1;11-15). The Focal Adhesion Targeting sequence located in the carboxy end of FAK, is responsible for the localization of FAK to focal adhesions (16;17). A FERM domain encompasses the amino terminus of FAK (18;19). FERM domains mediate protein-protein interactions. FAK has been found to mediate a variety of normal cellular processes including cell motility, cell proliferation, and cell survival (20-24). The deregulation of any of these cellular processes can lead to phenotypes characteristic of transformation and cancer.

FAK was first identified as a hyperphosphorylated protein in Src transformed cells (25). Interestingly, activation of Src and changes in integrin expression are common events in cancer (Gabarra-Niecko et al., 2003). Furthermore, FAK has been found to be overexpressed in a variety of tumors (26). The evidence up to date suggests a potential role for FAK in the initiation and progression of cancer (Reviewed in (26)). Thus, the aberration of FAK signaling is hypothesized to contribute to the process of oncogenic transformation. The purpose of this study was to determine the role of FAK signaling in the acquisition and regulation of cancer phenotypes in breast cancer epithelial cells. For this purpose, FAK signaling was enhanced in normal breast epithelial cells, MCF10A, by overexpression of wild type FAK or a hyperactive mutant of FAK, SuperFAK, which was constructed during the course of this work (27)(APPENDIX B). In addition, FAK was inhibited in a breast cancer epithelial cell line, T47D, by expressing a naturally occurring FAK dominant negative, FRNK (28). Biochemical and biological studies were performed to determine if FAK signaling could successfully be controlled in our cell model system. The normal and

breast cancer cells were monitored for changes in biological processes characteristic of cancer, including cell motility, cell proliferation, cell survival, adhesion-independent growth, invasion and tumor formation in mice.

BODY

In order to increase FAK signaling in normal breast epithelial cells, wild type FAK and SuperFAK, a hyperactive mutant of FAK (Gabarra-Niecko et al., 2002; APPENDIX B) were expressed in MCF10A cells, as previously reported. It was originally proposed to establish stable MCF10A cell lines expressing the different FAK proteins. However, we were unsuccessful in creating a cell line. Instead we took advantage of an amphotrophic retrovirus to infect and express wild type FAK and SuperFAK in MCF10A cells (APPENDIX A – Fig. 1A). A population of FAK and SuperFAK expressing MCF10A was produced as described in previous reports.

In order to express FAK, SuperFAK, and the FAK dominant negative, FRNK, in T47D cell, an avian retroviral receptor, Tva, was stably expressed on the surface of the T47D cells, as previously reported (APPENDIX A – Fig. 1B, and APPENDIX B & E). FAK, SuperFAK and FRNK were previously reported to be cloned into the RCAS A vector encoding for a replication competent avian retrovirus. Upon successful expression of the FAK proteins in chicken embryo fibroblasts, the cells produce and shed retrovirus encoding for the FAK proteins. These avian retroviruses can be collected and used to infect the T47D breast epithelial cells that stably express the retroviral receptor, Tva (APPENDIX A – Fig. 2). Successful expression of FAK, SuperFAK and FRNK in T47D/Tva cells has previously been reported (APPENDIX B & E).

Biochemical and biological studies were performed to ensure that FAK, SuperFAK and FRNK expression were behaving as expected, and thus would be valuable molecular tools to investigate the effect of aberrant FAK signaling in carcinogenesis. As previously reported, small expression of FAK and SuperFAK slightly increases cellular phosphotyrosine content, most notably of the FAK substrate, paxillin. Populations of MCF10A cells expressing higher levels of FAK and SuperFAK lead a dramatic increase in cellular phosphotyrosine as observed by Western blotting (APPENDIX A – Fig. 1B). However, there

was no significant change in the level of phosphotyrosine between FAK and SuperFAK expressing MCF10 cells (APPENDIX A – Fig. 1B). No further biochemical analyses were performed since no conclusive effects of FAK nor SuperFAK on adhesion independent growth were obtained (also discussed below).

As reported previously, SuperFAK had increased *in vitro* kinase activity compared to wild type FAK immunoprecipitated from T47D/Tva cells (APPENDIX E – Fig. 3-1). In order to determine if the expression of FAK, SuperFAK or FRNK was having an effect on FAK biochemical signals, the level of phosphorylation of FAK substrates, paxillin and p130cas, was determined by immunoprecipitation of each substrate followed by phosphotyrosine Western blotting (APPENDIX E – Fig. 3-2). FAK expression in T47D/Tva cells leads to very small increase in paxillin phosphorylation (APPENDIX E – Fig. 3-2 B). No further increase in paxillin phosphorylation was observed in SuperFAK expressing T47D/Tva cells (APPENDIX E – Fig. 3-2 B). However, a dramatic decrease in paxillin phosphorylation was observed in FRNK expressing T47D/Tva cells (APPENDIX E – Fig. 3-2 B). A similar effect of FRNK on paxillin phosphorylation was previously reported upon T47D/Tva cell adhesion to collagen, a physiological stimulus for FAK. In the case of p130cas phosphorylation, the most significant observation was an increase in its phosphorylation upon expression of SuperFAK in T47D/Tva cells (APPENDIX E – Fig. 3-2 C).

FAK is a known mediator of motility. Thus, to determine if increasing or decreasing FAK signaling with the molecular tools at hand could also affect biological processes mediated by FAK, the haptotactic motility of T47D/Tva cells expressing FAK, SuperFAK and FRNK was determined (APPENDIX B – Fig. 10; APPENDIX E – Fig. 3-3). As previously reported, FAK increased T47D/Tva cell motility and SuperFAK increases motility to higher levels (APPENDIX B – Fig. 10; APPENDIX E – Fig 3-3). In contrast, FRNK, the FAK dominant negative, was also reported to inhibit T47D/Tva cell motility in response to collagen (APPENDIX E – Fig. 3-3). These observations demonstrate that FAK biochemical and biological signals can be altered in T47D/Tva cells, and somewhat in MCF10, upon expression of FAK, SuperFAK and FRNK.

Upon alteration of FAK signaling, both normal (MCF10A) and cancer (T47D) breast epithelial cells were monitored for the acquisition or loss of cancer phenotypes to determine the role of FAK signaling in oncogenic trasnformation. As previously reported, FAK and SuperFAK alone had no effect on the ability of MCF10A cells to grow in an adhesion independent manner as measured using a soft agar growth assay. In past reports, it was also shown that in the presence of high levels of epidermal growth factor in the soft agar growth assay, FAK and SuperFAK expressing T47D/Tva cells but not mock transfected cells, acquired the ability to form colonies (adhesion independent growth). No difference in the number or size of the colonies was observed between FAK and SuperFAK expressing MCF10A. These results were shown in previous progress reports. Although these results seem promising we have been unable to reproduce these effects, suggesting that FAK and SuperFAK alone might not have an effect on MCF10 transformation. Considering the possible crosstalk between FAK and growth factor receptors (26;29) and the link that exists between growth factors and breast cancer, an additional collaborative signal i.e growth factor, may lead to the acquisition and/or enhancement of cancer phenotypes. However, further experimantion beyond the realm of this study is required to confirm this hypothesis, which might be very valuable in the quest to understanding breast carcinogenesis.

It was previously reported and is discussed in APPENDIX E, that FRNK inhibits T47D/Tva colony formation by 40% (APPENDIX E – Fig. 3-4). No significant changes in the number of colonies formed was observed upon FAK or SuperFAK expression (previously reported). In order to understand the molecular mechanism(s) through which FRNK can affect T47D/Tva adhesion independent growth,. a series of FRNK mutants were expressed in T47D/Tva cells and their effect on soft agar colony formation was determined (30)(APPENDIX E – Fig. 3-5). As shown and discussed in APPENDIX E, neither paxillin phosphorylation nor dominant negative activity (as measured by the ability of the FRNK mutants to block endogenous FAK phosphorylation) were critical determinants for the ability of FRNK to inhibit T47D/Tva colony formation. Furthermore, focal adhesion localization was not necessary for the inhibitory effect of FRNK either, as demonstrated by FRNK ML, a FRNK mutant that does not localize to focal adhesions (APPENDIX E – Fig. 3-5)(30).

The possibility that FRNK was impinging on cellular processes regulated by FAK could explain the mechanism through which FRNK inhibits T47D/Tva adhesion independent

growth. As previously reported and discussed in APPENDIX E, FRNK had no effect on the survival of T47D/Tva cells in culture or when grown in suspension (APPENDIX E – Fig. 3-7). These observations indicate that FAK signaling might not be a critical mediator of survival in T47D/Tva cells and that FRNK is not acting through a survival pathway to affect the ability of T47D/Tva cells to form colonies in soft agar. One characteristic phenotype of cancer cells is their ability to maintain high levels of proliferation (31). The rate of proliferation of T47D/Tva cells expressing FAK, SuperFAK or FRNK was determined (APPENDIX E – Fig. 3-6). As shown and discussed in APPENDIX E, neither FAK, SuperFAK nor FRNK had any effect on the growth ability of T47D/Tva cells grown in culture or in suspension (APPENDIX E – Fig. 3-6 A and B). Furthermore, an additional characteristic of cancer cells is their ability to continue growing even when growth signals are turned off or reduced (31). Neither FAK, SuperFAK, nor FRNK had an effect on the proliferation of T47D cells growing in low serum levels (APPENDIX E – Fig. 3-6 C). Thus, not only is FAK signaling not critical for the proliferation of the T47D/Tva cancer cells, but FRNK was not having any effect on the proliferation of the cells, suggesting that this might not be a mechanism through which FRNK inhibits T47D/Tva adhesion independent growth.

One of the most dramatic characteristics of cancer cells is their ability to form tumors when injected into athymic nude mice. The role of FAK signaling in the tumor formation of T47D/Tva cells was examined (APPENDIX E – Fig. 3-8). T47D/Tva cells expressing FAK, SuperFAK or FRNK were injected subcutaneously in the flank of female nude mice, as discussed in APPENDIC E. At the end of the *in vivo* tumorigenic study, no significant effect of FRNK nor FAK was observed. However, the presence of SuperFAK in the T47D/Tva cells was sufficient to potentiate their *in vivo* growth as demonstrated by an increase in the average tumor size (APPENDIX E – Fig. 3-8). Furthermore, analysis of tumor invasion suggested that none of the FAK proteins had any effect on the invasive ability of the T47D/Tva tumors. These observations suggest an important role for FAK in tumor growth.

As a side project, the ability of the FAK N-terminus to mediate an intramolecular interaction with the catalytic domain of FAK was examined and demonstrated (APPENDIX D). Although, this work was not initially proposed as part of this study its performance was warranted. The N-terminus contains the FERM domain, which mediates the molecular

association between FAK and growth factors. Considering the hypothesis that FAK and growth factor cooperate to regulate tumor initiation and progression, the normal mechanism of action of the FERM domain of FAK is of great importance. Understanding the function and molecular mechanism through which FAK could crosstalk to growth factor signaling is critical to studying the possible role of this crosstalk signaling in normal FAK function and in carcinogenesis.

KEY RESEARCH ACCOMPLISHMENTS

TASK 1

- Expression of FAK and SuperFAK in MCF10A cells
- Expression of FAK, SuperFAK, FRNK and FRNK mutants in T47D/Tva cells

TASK 2

- Increased paxillin phosphorylation by FAK in MCF10A cells.
- Decreased paxillin phosphorylation by FRNK in T47D/Tva cells.
- Increased p130cas phosphorylation by SuperFAK in T47D/Tva cells.
- Increased motility of T47D/Tva cells expressing FAK or SuperFAK.
- Decreased motility of T47D/Tva expressing FRNK.

TASK 3

- Decreased soft agar colony formation of T47D/Tva cells expressing FRNK.
- No effect of FAK or SuperFAK in T47D/Tva colony formation.
- No effect of FAK, SuperFAK nor FRNK in the ability of T47D/Tva cells to survive in culture or in suspension.
- No effect of FAK, SuperFAK or FRNK on proliferation of T47D/Tva cells kept in culture, held in suspension, or cultured in low levels of serum factors.
- Increased tumor growth of T47D/Tva cells expressing SuperFAK *in vivo*.
- No effect of FAK or FRNK in T47D/Tva tumor growth.
- No effect of FAK, SuperFAK or FRNK in T47D/Tva tumor invasion *in vivo*.

REPORTABLE OUTCOMES (Years 2002-2003)

APPENDIX A

Data figures and legends.

APPENDIX B

Published Article.

"Characterization of an Activated Mutant of Focal Adhesion Kinase: SuperFAK"

Veronica Gabarra-Niecko, Patricia J. Keely, Michael D. Schaller

Biochemical Journal 365: 591-603, 2002

APPENDIX C

Article "In Press".

"FAK regulates biological processes important for the pathogenesis of cancer"

Veronica Gabarra-Niecko, Michael D. Schaller, Jill M. Dunty

Cancer and Metastasis Reviews 22: 359-374, 2003

APPENDIX D

Manuscript submitted to Molecular and Cellular Biology.

"Interaction of the FERM and Catalytic Domains of FAK Promotes FAK Signaling"

Jill M. Dunty*, Veronica Gabarra-Niecko*, Michelle L. King, Michael D. Schaller

* The first two authors contributed equally to this work.

APPENDIX E

Manuscript in Progress.

"The Role of FAK Signaling in Tumorigenesis of Breast Epithelial Cells"

Veronica Gabarra-Niecko and Michael D. Schaller

APPENDIX F

Era of Hope. Department of Defense Breast Cancer Research Program Meeting.

Orange County Convention Center, Orlando, Florida

September 25-28, 2002

APPENDIX G

Degree Obtained.

Veronica Gabarra-Niecko succesfully defended her Ph.D on April 1, 2003

CELL LINES:

MCF10A expressing FAK and SuperFAK

T47D/Tva cell line

T47D/Tva cells expressing FAK, SuperFAK, FRNK, HK, ML, TRP

CONCLUSIONS

The main goals of this work have been to develop the appropriate molecular tools and a cell model system to begin to investigate the role of FAK signaling in cancer initiation and progression, and to explore the molecular mechanisms by which FAK might signal in the pathology of cancer. The first aim of the project was to engineer hyperactive mutants of FAK that could be used to mimic FAK overexpression in cancer cells. To this end, a hyperactive mutant of FAK, SuperFAK, was succesfully developed. SuperFAK has increased kinase activity in culture and suspension compared to wild type FAK, and elevates FAK substrate phosphorylation, and cell motility to higher levels than FAK. Importantly, SuperFAK is regulated like wild type FAK and localizes to the correct cellular compartment. Conversely, in order to block FAK signals we took advantage of a naturally occurring dominant negative of FAK, FRNK. Thus, we successfully engineered and identified molecular tools that could be used to manipulate biochemical and biological FAK signaling in cells.

The human T47D breast epithelial cancer cell line was chosen as our cancer cell model system. These cells exhibit adhesion independent growth and tumor formation in mice. Stable expression of an avian retroviral receptor in these cells enabled expression

of various FAK constructs using a replication competent retroviral vector, allowing for successful manipulation of biochemical and biological signals controlled by FAK. For example, the introduction of SuperFAK or FRNK led to an increase or reduction respectively in T47D cell motility in a transwell assay system.

The first study using the T47D/Tva cell model system has already revealed important aspects about FAK signaling in cancer. Inhibition of FAK signaling led to a decrease in T47D adhesion independent growth. Furthermore, augmentation of FAK signals in T47D cells increases tumor growth. The observations of this first study demonstrate not only the value of our system, but also indicate an important role for FAK signaling in the regulation of cancer phenotypes. At this point we can begin to determine the mechanism of action of FAK in cancer and its role in cooperating with additional oncogenic signals.

The initial efforts to understand FAK mediated regulation of cancer phenotypes took advantage of a series of FRNK mutants. Using the soft agar assay it was shown that FRNK efficiently reduces adhesion independent growth in T47D cells. FA localization and paxillin binding were dispensable for the ability to block soft agar growth. However, FRNK also contains binding sites for talin, GRAF and p130cas. Since p130cas is a known substrate of the Src oncogene, it may be interesting to determine if p130cas binding to FRNK may be a possible mode of action for the inhibition of adhesion independent growth. The initial observations in this study suggest a potential role of FAK in the regulation of cancer.

REFERENCES

1. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T. (1992) *Proc.Natl.Acad.Sci.U.S.A* **89,** 5192-5196

2. Schneider, G. B., Gilmore, A. P., Lohse, D. L., Romer, L. H., and Burridge, K. (1998) *Cell Adhes.Commun.* **5,** 207-219

3. Schaller, M. D. (2001) *Biochim.Biophys.Acta* **1540,** 1-21

4. Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R., and Parsons, J. T. (1994) *Mol.Cell Biol.* **14,** 1680-1688

5. Cobb, B. S., Schaller, M. D., Leu, T. H., and Parsons, J. T. (1994) *Mol.Cell Biol.* **14,** 147-155

6. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der, G. P. (1994) *Nature* **372,** 786-791

7. Schlaepfer, D. D. and Hunter, T. (1996) *Mol.Cell Biol.* **16,** 5623-5633

8. Chen, H. C. and Guan, J. L. (1994) *Proc.Natl.Acad.Sci.U.S.A* **91,** 10148-10152

9. Calalb, M. B., Polte, T. R., and Hanks, S. K. (1995) *Mol.Cell Biol.* **15,** 954-963

10. Schlaepfer, D. D. and Hunter, T. (1996) *Cell Struct.Funct.* **21,** 445-450

11. Thomas, J. W., Ellis, B., Boerner, R. J., Knight, W. B., White, G. C., and Schaller, M. D. (1998) *J.Biol.Chem.* **273,** 577-583

12. Pawson, T. and Gish, G. D. (1992) *Cell* **71,** 359-362

13. Harte, M. T., Hildebrand, J. D., Burnham, M. R., Bouton, A. H., and Parsons, J. T. (1996) *J.Biol.Chem.* **271,** 13649-13655

14. Polte, T. R. and Hanks, S. K. (1995) *Proc.Natl.Acad.Sci.U.S.A* **92,** 10678-10682

15. Cary, L. A., Han, D. C., Polte, T. R., Hanks, S. K., and Guan, J. L. (1998) *J.Cell Biol.* **140,** 211-221

16. Hildebrand, J. D., Schaller, M. D., and Parsons, J. T. (1993) *J.Cell Biol.* **123,** 993-1005

17. Hildebrand, J. D., Schaller, M. D., and Parsons, J. T. (1995) *Mol.Biol.Cell* **6,** 637-647

18. Girault, J. A., Labesse, G., Mornon, J. P., and Callebaut, I. (1999) *Trends Biochem.Sci.* **24,** 54-57

19. Chishti, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N., Chasis, J. A., Conboy, J. G., Gascard, P., Takakuwa, Y., Huang, S. C., Benz, E. J., Jr., Bretscher, A., Fehon, R. G., Gusella, J. F., Ramesh, V., Solomon, F., Marchesi, V. T., Tsukita, S., Tsukita, S., Hoover, K. B., and . (1998) *Trends Biochem.Sci.* **23,** 281-282

20. Gilmore, A. P. and Romer, L. H. (1996) *Mol.Biol.Cell* **7,** 1209-1224

21. Hungerford, J. E., Compton, M. T., Matter, M. L., Hoffstrom, B. G., and Otey, C. A. (1996) *J.Cell Biol.* **135,** 1383-1390

22. Frisch, S. M., Vuori, K., Ruoslahti, E., and Chan-Hui, P. Y. (1996) *J.Cell Biol.* **134,** 793-799

23. Romer, L. H., McLean, N., Turner, C. E., and Burridge, K. (1994) *Mol.Biol.Cell* **5,** 349-361

24. Zhao, J. H., Reiske, H., and Guan, J. L. (1998) *J.Cell Biol.* **143,** 1997-2008

25. Kanner, S. B., Reynolds, A. B., Vines, R. R., and Parsons, J. T. (1990) *Proc.Natl.Acad.Sci.U.S.A* **87,** 3328-3332

26. Gabarra-Niecko, V., Schaller, M. D., and Dunty, J. M. (2003) *Cancer Metastasis Rev.* **22,** 359-374 (In Press)

27. Gabarra-Niecko, V., Keely, P. J., and Schaller, M. D. (2002) *Biochem.J.* **365,** 591-603

28. Richardson, A. and Parsons, T. (1996) *Nature* **380,** 538-540

29. Dunty, J. M., Gabarra-Niecko, V., and Schaller, M. D. (2003) *Mol.Cell Biol.* Submitted

30. Cooley, M. A., Broome, J. M., Ohngemach, C., Romer, L. H., and Schaller, M. D. (2000) *Mol.Biol.Cell* **11,** 3247-3263

31. Hanahan, D. and Weinberg, R. A. (2000) *Cell* **100,** 57-70

APPENDICES

APPENDIX A

2003 Annual Summary Report Figures and Legends

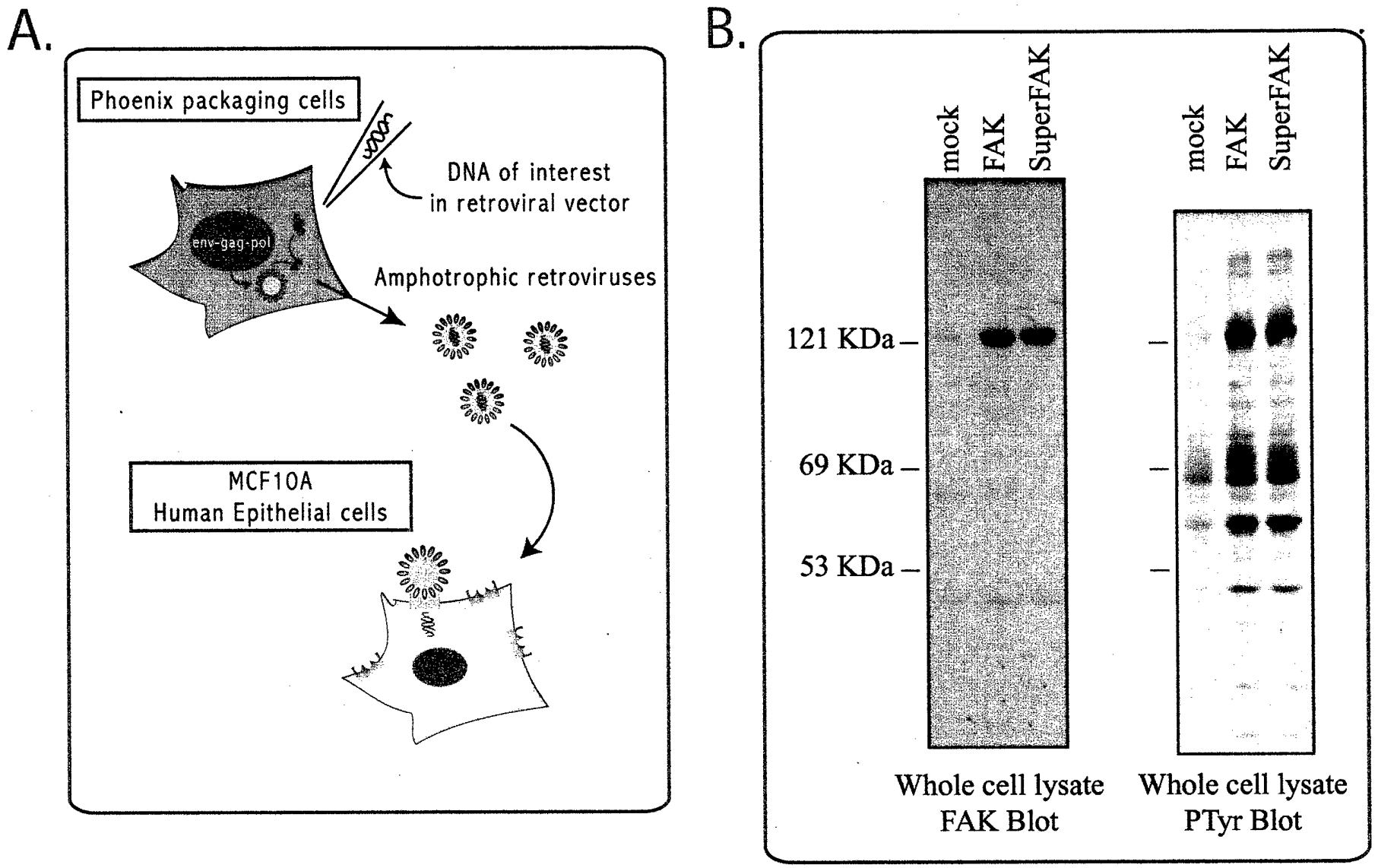

Figure 1: Expression in MCF10A, Normal immortalized Breast Epithelial Cells

(**A**) A packaging cell line was used to produce retroviruses encoding FAK and SuperFAK. MCF10A cells were infected with the retrovirus to achieve expression of FAK or SuperFAK. A population of successfully infected cells was isolated. (**B**) To determine FAK and SuperFAK expression, whole cell lysates (25 μγ) of MCF10A infected with the retroviruses were Western blotted with a polyclonal FAK antibody (left panel). Whole cell lysates of MCF10A cells expressing FAK or SuperFAK were also Western blotted with a phosphotyrosine antibody (right panel).

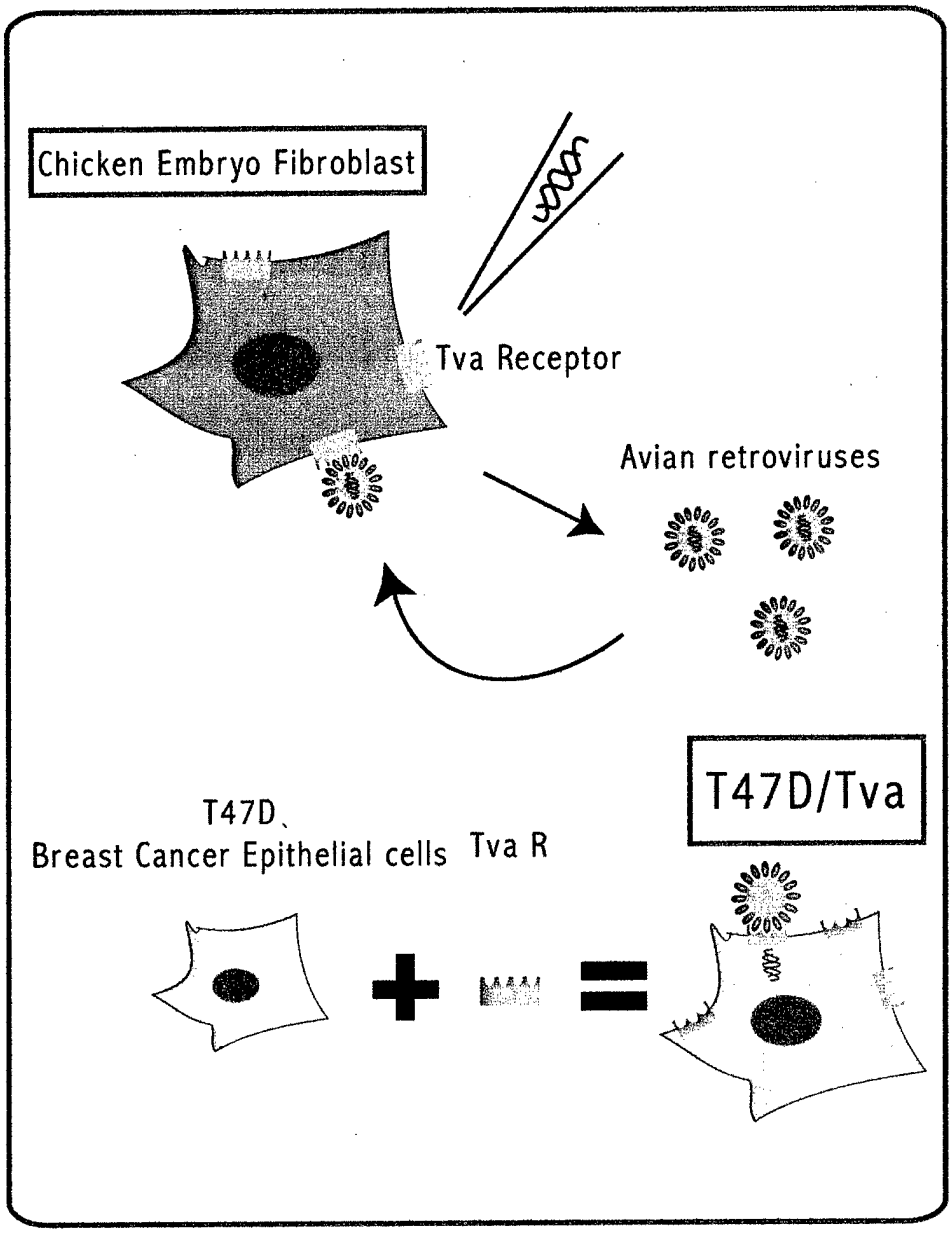

Figure 2: T47D/Tva Breast Carcinoma Cell Expression System

T47D cells stably expressing the avian retroviral receptor (Tva) were infected with the avian retrovirus produced by chicken embryo fibroblasts expressing the constructs of interest.

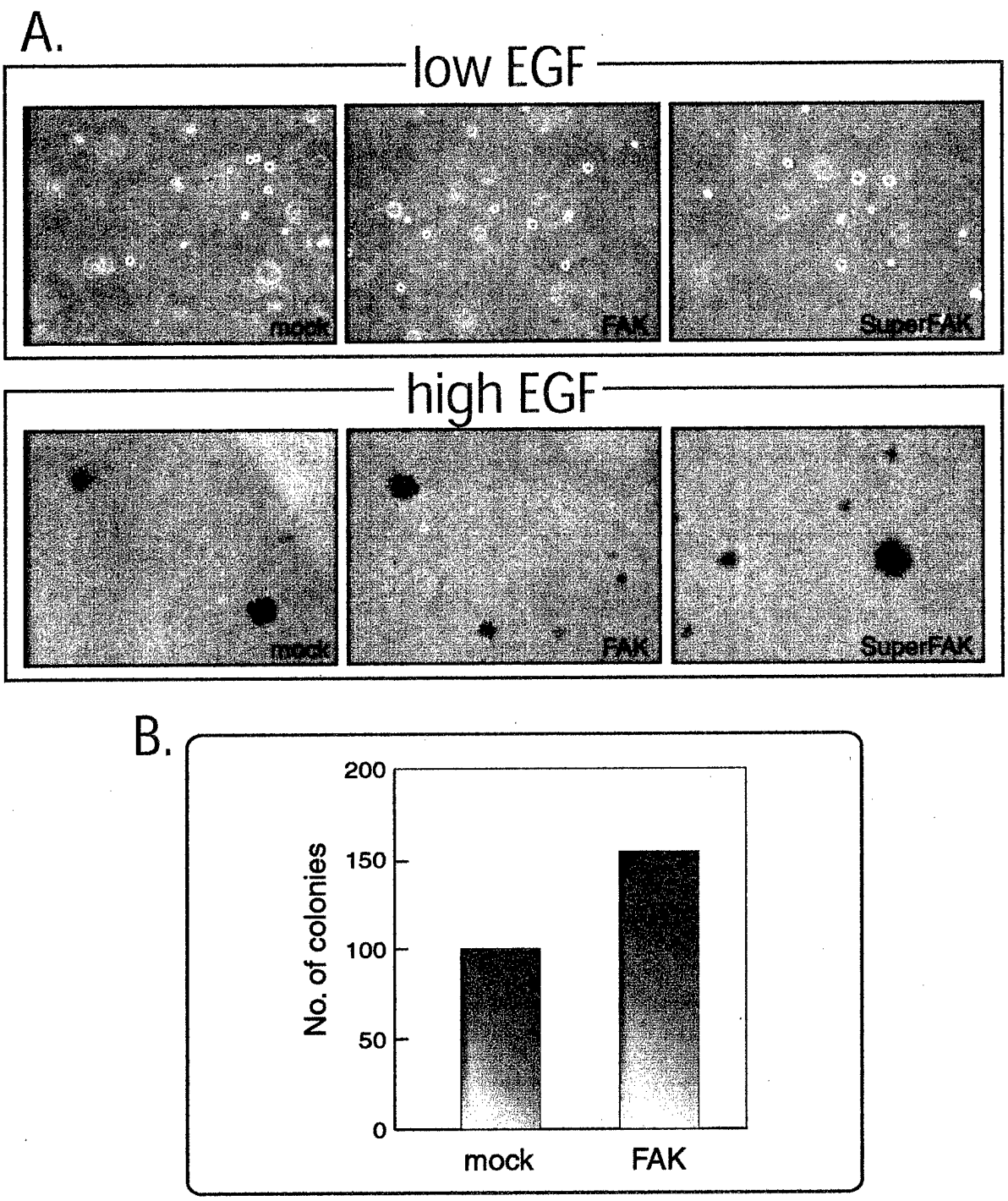

Figure 3: Expression in MCF10A, Normal immortalized Breast Epithelial Cells

(**A**) A packaging cell line was used to produce retroviruses encoding FAK and SuperFAK. MCF10A cells were infected with the retrovirus to achieve expression of FAK or SuperFAK. A population of successfully infected cells was isolated.

(**B**) To determine FAK and SuperFAK expression, whole cell lysates (25 μγ) of MCF10A infected with the retroviruses were Western blotted with a polyclonal FAK antibody (left panel). Whole cell lysates of MCF10A cells expressing FAK or SuperFAK were also Western blotted with a phosphotyrosine antibody (right panel).

APPENDIX B

Published Article.

"Characterization of an Activated Mutant of Focal Adhesion Kinase: SuperFAK"

Characterization of an activated mutant of focal adhesion kinase: 'SuperFAK'

Veronica GABARRA-NIECKO*, Patricia J. KEELY† and Michael D. SCHALLER*‡[1]

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Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that plays an important role in normal cellular processes such as adhesion, spreading, migration, proliferation and survival. In addition, FAK is overexpressed in a variety of cancer cells and tumours and may play a role in the development of human cancer. As a prelude to modelling the role of aberrant FAK signalling in the initiation of cancer, the goal of the present study was to engineer point mutations in FAK that would enhance enzymic activity. A number of substitutions that were reported as activating mutations in other tyrosine kinases were introduced into FAK. Glutamic acid substitutions for two lysine residues in the activation loop of FAK, based upon the K650E (Lys^{650}→Glu) mutant of fibroblast-growth-factor receptor 3, were made to create 'SuperFAK'. Two brain-specific exons were engineered into avian FAK to create FAK6.7. SuperFAK and, to a lesser extent, FAK6.7, exhibited increased catalytic activity *in vitro* compared with wild-type FAK. The expression of SuperFAK and FAK6.7 in fibroblasts led to hyperphosphorylation of FAK substrates. Although the catalytic activity of SuperFAK and FAK6.7 was largely independent of cell adhesion, tyrosine phosphorylation of downstream substrates was adhesion-dependent. Further, since SuperFAK exhibited the same ability as wild-type FAK to recruit Src family kinases, tyrosine phosphorylation of substrates was likely due to direct phosphorylation by FAK. In addition to enhanced biochemical signalling, SuperFAK also increased the motility of epithelial cells. SuperFAK and FAK6.7 may be valuable molecular tools to investigate the potential role of aberrant FAK signalling in human disease.

Key words: integrin, motility, phosphotyrosine, paxillin, Src.

INTRODUCTION

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase, first identified in Src-transformed fibroblasts [1]. FAK localizes to focal adhesions through its focal-adhesion-targeting (FAT) sequence located at the C-terminus [2]. The clustering of integrins at focal adhesions upon engagement of their extracellular-matrix (ECM) protein ligands results in tyrosine phosphorylation and activation of FAK [1]. In addition to adhesion, treatment of cells with a variety of soluble factors, including G-protein-coupled-receptor agonists and receptor protein tyrosine kinase ligands, can also induce FAK activation [1].

Upon activation, FAK autophosphorylates on Tyr^{397} [3,4], creating a binding site for SH2-domain-containing proteins. The p85 regulatory subunit of phosphoinositide 3-kinase (PI 3-kinase; 'phosphatidylinositol 3-kinase'), phospholipase C-γ1 (PLC-γ1), growth-factor-receptor-bound protein 7 (Grb7), and possibly Src-homology (SH)-containing protein (Shc), bind to phosphorylated Tyr^{397} through SH2-mediated interactions [5–8]. In addition, the autophosphorylation site on FAK recruits Src-like kinases via their SH2 domains [4]. Furthermore, FAK contains a proline-rich sequence upstream of Tyr^{397}, which facilitates binding to the SH3 domain of Src and stabilization of the FAK–Src complex [9,10]. Once Src binds to FAK, it phosphorylates additional tyrosine residues on FAK, including Tyr^{576}, Tyr^{577} and Tyr^{925} [3,11]. Phosphorylation of Tyr^{576} and Tyr^{577}, which reside in the activation loop of FAK, lead to maximal activation of FAK [3]. Phosphorylation of Tyr^{925} creates a binding site for the SH2 domain of Grb2, which may link FAK to mitogen-activated protein kinase (MAPK) signalling ([11,12], but see [12a]). In addition to SH2-binding sites, FAK also contains proline-rich regions that serve as docking sites for SH3-containing proteins, including the crk-associated protein $p130^{cas}$ [13,14]. FAK can thus recruit a variety of signalling proteins to form an intricate signalling complex.

FAK is implicated in controlling a variety of integrin-mediated biological processes. FAK regulates turnover of focal adhesions, apparently by regulating the activity of Rho ([15], but see [15a]; [16,17]). FAK also regulates cell motility. FAK-null fibroblasts and cells expressing a dominant-negative form of FAK show decreased migration [16,18]. Conversely, the overexpression of FAK in Chinese-hamster ovary (CHO) cells increases cell motility [19]. The autophosphorylation site of FAK is required for the regulation of cell motility [19] and two effectors, PI 3-kinase and Src kinases, have been shown to function in the FAK-dependent regulation of cell motility [6,19]. Furthermore, $p130^{cas}$ has been implicated as a downstream component of the FAK-mediated signalling pathway controlling motility [20]. In addition to migration, FAK also plays a role in mediating cell survival. Inhibition of FAK signalling causes cells to undergo apoptosis [21,22]. Furthermore, a constitutively activated FAK-containing chimaeric protein, CD2FAK, is able to rescue cells held in suspension from undergoing anoikis [23]. Recently FAK overexpression was shown to inhibit apoptosis

Abbreviations used: CE, chicken embryo; CHO, Chinese-hamster ovary; ECM, extracellular matrix; EGFR, epidermal-growth-factor receptor; ERK, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; FAT, focal adhesion targeting (sequence); FGFR3, fibroblast-growth-factor receptor type 3; Grb, growth-factor-receptor-bound; GST, glutathione S-transferase; K650E, Lys^{650}→Glu; MAPK, mitogen-activated protein kinase; $p130^{cas}$, p130 crk-associated substrate; PI 3-kinase, phosphoinositide 3-kinase ('phosphatidylinositol 3-kinase'); PLC-γ1, phospholipase C-γ1; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine; PY, phosphotyrosine; RIPA, radioimmunoprecipitation; SH, Src homology; Shc, Src-homology-containing protein; SuperFAK, an activated K578E/K581E mutant of FAK; TBS, Tris-buffered saline.

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induced by other stimuli [24,25]. Roles for PI 3-kinase, p130^{cas} and Grb2 in FAK-mediated cell survival have been proposed [25,26]. In addition to regulating cell survival, FAK may also function in the positive regulation of the cell cycle by controlling the levels of cyclin D and the cyclin-dependent kinase inhibitor p21 [27]. Although some of the mechanisms of action are not fully understood, FAK transduces important biological signals following integrin-dependent cell adhesion.

Constitutively activated FAK variants have been described previously [28,29]. However, activation has been achieved by targeting FAK constitutively to the cell membrane. Since FAK is not membrane-bound, these variants may have acquired novel properties and may not fully mimic FAK. Therefore the creation of an activated mutant of FAK exhibiting proper cellular localization is of special interest. A number of reports in the literature describe activating point mutations in tyrosine kinases. These include a valine-to-isoleucine mutation in the ATP-binding pocket of the epidermal-growth-factor receptor (EGFR) [30], a methionine-to-threonine mutation in the C-terminal lobe of the catalytic domain of RET, Met/HGF/SFR, Ron/RMSH, and Kit kinases [31–34], and substitution of a glutamic acid for a lysine residue in the activation loop of the fibroblast-growth-factor receptor type 3 (FGFR3) [35]. In the present study we describe the construction and characterization of activated mutants of FAK. Point mutations shown to activate other tyrosine kinases were engineered into FAK. In addition, the regulatory tyrosine residues in the activation loop of FAK, Tyr^{576} and Tyr^{577}, were mutated to glutamic acid to potentially mimic their phosphorylation. Finally, an avian version of FAK6.7, a neuronal FAK variant with two inserts flanking the autophosphorylation site of FAK that exhibits high autophosphorylation activity, was also engineered [36]. Two of the mutants, SuperFAK (with glutamic acid for lysine substitutions in the activation loop) and FAK6.7, exhibited elevated catalytic activity compared with wild-type FAK. Furthermore, expression of these mutants led to the hyperphosphorylation of the FAK substrates tensin and paxillin as well as FAK itself. Strikingly, upon loss of adhesion, substrate phosphorylation disappeared in SuperFAK and FAK6.7 overexpressors, despite the fact that the kinase activity of the mutants remained high. The increased signalling capacity of SuperFAK and FAK6.7 occurred without affecting FAK–Src complex formation or Src activation, implicating FAK activity itself in the augmentation of downstream signalling. In addition to enhanced biochemical signalling, the activated mutant SuperFAK was also able to enhance biological signals, since expression of SuperFAK increased cell motility in T47D breast epithelial cells. These activated FAK mutants may be powerful molecular tools for investigating the potential role of FAK signalling in the pathology of human disease, including cancer.

EXPERIMENTAL

Cloning and mutagenesis

In order to make an avian version of FAK6.7, the codons for the 6 (^{393}DEISGD^{398}) and 7 (^{412}KSYGIDE^{418}) amino acid inserts were introduced into the avian FAK cDNA by site-directed mutagenesis using the Altered Sites Mutagenesis Kit (Promega, Madison, WI, U.S.A.). Mutants were identified by PCR amplification and nucleotide sequencing. The *Msc*I–*Sal*I fragment of the mutated cDNA (extending from nucleotide 1178 in FAK to the multiple cloning sequence of the vector, downstream of the FAK stop codon) was excised from the mutagenesis vector, pALTER, and substituted for the corresponding fragment of wild-type FAK in pBluescript-FAK [37]. Point mutations were engineered into the full-length avian FAK cDNA in pBluescript-FAK [37] by oligonucleotide-directed PCR mutagenesis using the Stratagene QuikChange kit (Stratagene, La Jolla, CA, U.S.A.). Some mutants were initially identified by restriction digestion. To verify the presence of the intended mutations and that no unintended mutations were introduced during the mutagenesis procedures, each construct was completely sequenced at the UNC-CH Automated DNA Sequencing Facility on a model 377 DNA Sequencer (PerkinElmer, Applied Biosystems Division) using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (PerkinElmer, Applied Biosystems Division). The full-length mutant FAK cDNAs were subcloned into the replication-competent avian retroviral vector RCAS type A. RCAS A-FAK and RCAS B-c-Src constructs have already been described [2,38,39].

Cells and viruses

Chicken embryo (CE) cells were harvested from 9-day-old embryos and grown as previously described [40]. T47D breast epithelial cells and the T47D/Tva derivatives were maintained in RPMI 1640 (Gibco BRL, Rockville, MD, U.S.A.) supplemented with 10% (v/v) fetal-bovine serum (Gibco BRL), 0.2 unit/ml insulin (Gibco BRL), penicillin, streptomycin, genamycin and kanamycin (Sigma, St. Louis, MO, U.S.A.). CE cells were transfected with RCAS plasmid DNA using the LIPOFECTAMINE PLUS™ reagent (Gibco BRL) following the manufacturer's recommended protocol. At 7 days after transfection, cells were lysed and FAK expression was analysed. T47D cells were transfected with the avian retroviral receptor Tva800 cDNA (generously given by Dr P. Bates, Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A.) [41] using the Superfect reagent (Qiagen, Valencia, CA, U.S.A.). Cells were selected in G418-containing growth medium and expanded as stably transfected cells. Expression of constructs was determined by fluorescent immunolabelling using a polyclonal antibody to the Tva receptor (generously given by Dr P. Bates), and a secondary FITC-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA, U.S.A.), followed by fluorescence analysis through flow cytometry.

Viral stocks were made from subconfluent cultures of CE cells 10 days after transfection. The culture medium was removed, 4 ml of fresh culture medium was added, and the cells were incubated overnight. The culture medium was collected, cells and debris were pelleted by centrifugation, and virus-containing supernatants were divided into portions and stored at −70 °C. Upon passaging T47D cells, 1 ml of virus stock was added to the T47D cultures. At 10–14 days after infection, cells were lysed and FAK expression was analysed.

For adhesion experiments, cells were trypsinized and washed twice in PBS containing 0.5 mg/ml soybean trypsin inhibitor (Sigma). Cells were resuspended in serum-free medium and kept in suspension for 45 min at 37 °C. Suspended cells were then collected and lysed or plated at a concentration of 2.5×10^5 cells/ml on fibronectin-coated dishes (50 μg/ml) for the indicated times prior to lysis [41a].

Protein analysis

Cells were lysed in modified radioimmunoprecipitation (RIPA) buffer containing protease and phosphatase inhibitors as

previously described [42]. The protein concentration of the lysates was determined using the bicinchoninic acid ('BCA') assay (Pierce, Rockford, IL, U.S.A.).

For immunoprecipitations, 0.3–1 mg of cell lysate was incubated with primary antibody on ice for 1 h. The polyclonal FAK antibody BC4, monoclonal tensin antibody 5B9 (generously given by Dr Tom Parsons, Department of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA, U.S.A.), Fyn antiserum (kindly given by Dr André Veillette, Laboratory of Molecular Oncology, Institut de Recherches, Cliniques de Montreal, Montreal, PQ, Canada), the monoclonal Src antibody EC10 (generously given by Dr Sally Parsons, Department of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA, U.S.A.), paxillin and $p130^{cas}$ antibodies (Transduction Laboratories, Lexington, KY, U.S.A.) were used for immunoprecipitations. Immune complexes were precipitated with Protein A–Sepharose beads (Sigma) or rabbit anti-mouse IgG (Jackson Immunoresearch Laboratories) pre-bound to Protein A–Sepharose beads (Sigma) at 4 °C for 1 h. The immune complexes were then washed twice with modified RIPA buffer, and twice with Tris-buffered saline (TBS; 10 mM Tris/150 mM NaCl, pH 7.0). Immune complexes were denatured and dissociated from beads by boiling in Laemmli sample buffer [43]. The samples were then resolved by SDS/PAGE on an 8% gel, and analysed by Western blotting. Nitrocellulose membranes were blocked with TBS-T (10 mM Tris/150 mM NaCl, pH 7.0, containing 0.1% Tween 20) containing 5% (w/v) powdered milk or with TBS-T alone when using the RC20 phosphotyrosine antibody or with TBS-T containing 2% fish gelatin (Sigma) at 4 °C overnight when using the [$PTyr^{397}$]FAK phosphospecific antibody. Membranes were incubated with primary antibody in blocking solution for 1 h at room temperature. The antibodies described above were used for Western blotting. For detection of phosphotyrosine, horseradish peroxidase-conjugated RC20 (Transduction Laboratories) or polyclonal [$PTyr^{397}$]FAK, [Tyr^{416}]Src and [Tyr^{527}]Src phosphospecific antibodies (BioSource International, Camarillo, CA, U.S.A.) were used. Membranes were incubated overnight at 4 °C when using the [$PTyr^{397}$]FAK phosphospecific antibody. Primary antibodies were detected using horseradish peroxidase conjugated to Protein A or anti-mouse IgG and enhanced chemiluminescence (ECL®; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

In vitro kinase assays

For *in vitro* kinase reactions, FAK or Src immune complexes were washed twice in modified RIPA buffer, twice with TBS and once with kinase reaction buffer [20 mM Pipes (pH 7.2)/7.5 mM $MnCl_2$/2.5 mM $MgCl_2$] or enolase kinase buffer [20 mM Pipes (pH 7.2)/10 mM $MgCl_2$/1 mM dithiothreitol]. For enolase kinase assays, 5 μg of acid-denatured enolase (Sigma) was added to each reaction mixture. For treatments with 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), either 0.5 μM PP2 (Calbiochem, San Diego, CA, U.S.A.) or vehicle (DMSO; Fisher Scientific, Pittsburgh, PA, U.S.A.) alone were added to the kinase buffer. The immune complexes were then incubated in kinase buffer and 10 μCi of [γ-^{32}P]ATP (Dupont–NEN, Wilmington, DE, U.S.A.) at room temperature for the times indicated. The kinase reactions were stopped by boiling in Laemmli sample buffer [43]. The reactions were subjected to SDS/PAGE. The gels were fixed in 7% acetic acid and 20% methanol and dried. ^{32}P incorporation was visualized by autoradiography and PhosphorImager analysis using the Storm860 instrument (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

In vitro binding assays

The glutathione S-transferase (GST) fusion proteins were expressed in *Escherichia coli* and purified as described in [43a]. Briefly, expression was induced by the addition of 0.1 mM isopropyl 1-thio-β-D-galactopyranoside and incubation at 37 °C for 2 h. The bacteria were harvested and lysed by sonication in 1% Triton X-100 in PBS containing protease inhibitors (1 mM PMSF, 0.5 mM EDTA, pH 8.0, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Clarified supernatants were incubated with GSH–agarose beads (Sigma) for 1 h at 4 °C, washed, and finally resuspended in an equal volume of PBS. The fusion proteins were analysed by SDS/PAGE and Coomassie Blue staining.

Approx. 0.5–1 mg of protein lysate was pre-cleared by incubation with GST immobilized on GSH–Sepharose beads for 1 h at 4 °C. For GST–Grb2SH2 pulldowns, cells were treated overnight with 50 μM sodium orthovanadate before lysing. The cleared lysates were then incubated with 2 μg of GST alone or GST–SH2 domain fusion proteins immobilized on GSH–Sepharose beads for 2 h at 4 °C. The beads were washed twice with modified RIPA buffer and twice with TBS. The bound proteins were denatured and eluted from the beads by boiling in Laemmli sample buffer [43] and analysed by Western blotting.

Motility

Motility assays were performed as described previously [44]. The underside of 12 mm-diameter transwell chambers with a 12-μm-pore-size polycarbonate membrane (Costar, Cambridge, MA, U.S.A.) were coated with 0.6 ml of 40 μg/ml rat tail collagen I (Collaborative Biomedical Products, Bedford, MA, U.S.A.) for 6 h at 37 °C. The lower chamber was washed twice and filled with serum-free RPMI 1640 medium. T47D cells were trypsinized, counted, and resuspended in RPMI 1640 medium supplemented with 5 mg/ml BSA (Sigma) to a total concentration of 3×10^6 cells/ml. Then 1.5×10^6 cells were added to the top chamber of the transwell. The T47D cells were allowed to migrate for 20–22 h at 37 °C. Cells remaining on the top of the polycarbonate membrane were removed. Cells that had migrated to the underside of the membrane were stained with DiffQuick (Baxter, Miami, FL, U.S.A.). The cells were counted across two diameters, a total of ten fields, each on duplicate membranes. A mixed model test as well as paired and unpaired Student *t* tests were performed using the SAS™ (Cary, NC, U.S.A.) software to identify statistically significant differences in average fold change of motility.

RESULTS

A number of mutations known to activate tyrosine kinases were engineered into avian FAK (Figure 1). The Y576E ($Tyr^{576} \rightarrow$ Glu)/Y577E, V436I and M589T mutations had no effect on the kinase activity of FAK or signalling downstream of FAK (results not shown) and thus were not further characterized. The K578E/K581E mutant, referred to as SuperFAK, and the alternatively spliced neuronal form, FAK6.7, exhibited elevated catalytic activity and were more extensively characterized.

SuperFAK has increased catalytic activity compared with wild-type FAK

SuperFAK and FAK6.7 were subcloned into the RCAS A retroviral vector and expressed in CE cells. Western blotting of CE cell lysates with a polyclonal FAK antibody revealed that wild-type FAK, SuperFAK and FAK6.7 were expressed at equal levels (Figure 2A). The altered electrophoretic mobility of FAK6.7

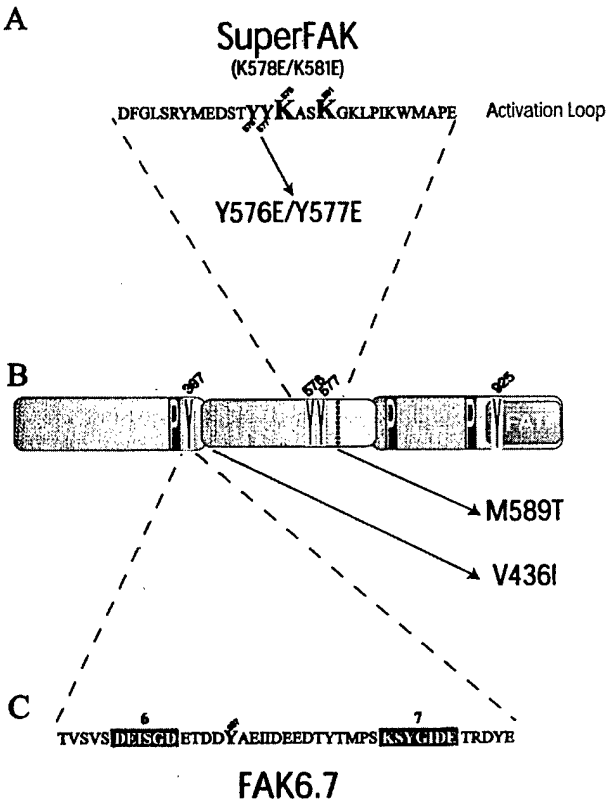

Figure 1 Construction of activated mutants of FAK

(**A**) The sequence of the activation loop of the kinase domain of FAK is shown. In SuperFAK two lysine residues, Lys[578] and Lys[581] (large Ks with diagonal numbers at the top) were substituted with glutamic acids (E), mimicking the activating K650E mutation in FGFR3 [53]. Y576E/Y577E represents an additional mutant in which the regulatory tyrosine residues (Tyr[576] and Tyr[577]) in the activation loop of FAK (bold **Y**s with diagonal numbers at the base) were mutated to glutamic acid (E). (**B**) A schematic diagram of FAK is shown. Proline-rich regions (white Ps on mid-grey background), sites of tyrosine phosphorylation (grey Ys on a white background) and the FAT (white 'FAT' on grey) sequence are shown. Two additional FAK mutants were engineered to mimic activating point mutations in EGFR (V436I; vertical white circles) [30] and RET, Met/HGF/SFR, Ron/RMSH, and Kit kinases (M589T; vertical black circles) [31–34]. (**C**) FAK6.7, an alternatively spliced neuronal form of FAK [36], contains two additional exons (6 and 7; grey boxes with white lettering) flanking Tyr[397], the autophosphorylation tyrosine residue on FAK.

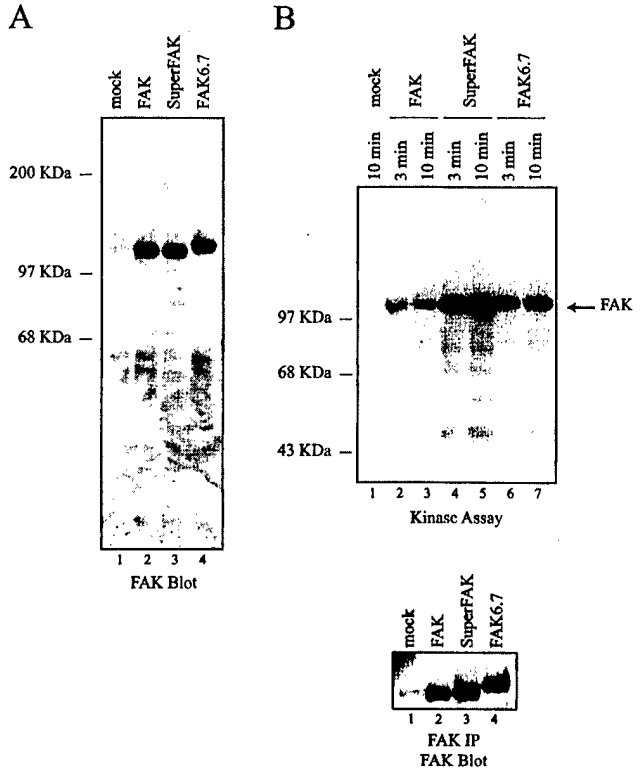

Figure 2 Expression and *in vitro* kinase activity

(**A**) Expression of FAK and the FAK mutants was detected by Western-blot analysis. At 7–10 days after transfection, lysates (25 μg) of CE cells expressing empty vector (lane 1), FAK (lane 2), SuperFAK (lane 3) or FAK6.7 (lane 4) were Western-blotted with a FAK polyclonal antibody, BC4. (**B**) The *in vitro* autophosphorylation activity of wild-type FAK and the FAK mutants was determined using an immune complex kinase assay. FAK was immunoprecipitated from CE cell lysates (0.5–1 mg) expressing vector alone (lane 1), FAK (lane 2), SuperFAK (lane 3) or FAK6.7 (lane 4), using a polyclonal FAK antibody, BC4. The immune complexes were incubated in kinase buffer containing [γ-^{32}P]ATP for 3 or 10 min. The samples were subjected to SDS/PAGE and visualized by autoradiography (top panel). The immune complexes were also Western-blotted using a polyclonal FAK antibody, BC4, to ensure equal loading (bottom panel). The positions of the molecular-mass markers are indicated on the left.

was due to the two insertions (Figure 2A, lane 4). Immunofluorescence studies demonstrated that, like wild-type FAK, both SuperFAK and FAK6.7 localized to focal adhesions (results not shown).

To determine the effect of the introduced mutations and insertions on catalytic activity, FAK, SuperFAK and FAK6.7 were subjected to *in vitro* kinase assays. The proteins were immunoprecipitated from CE lysates and then incubated in kinase reaction buffer for the indicated times. The kinase reactions were stopped by the addition of Laemmli sample buffer and analysed by SDS/PAGE and autoradiography [43]. Autophosphorylation of endogenous FAK was not detected at this exposure (Figure 2B; top panel; lane 1), due to the small amount of endogenous FAK that is recovered relative to the exogenously expressed proteins (Figure 2B; bottom panel). However, autophosphorylation of exogenous wild-type FAK was readily detected (Figure 2B; top panel, lanes 2 and 3). A significant increase in autophosphorylation activity was observed in SuperFAK immune complexes compared with wild-type FAK (Figure 2B; top panel, lanes 4 and 5). FAK6.7 also exhibited increased autophosphorylation activity relative to wild-type FAK, but the increase was less dramatic than that of SuperFAK (Figure 2A, top panel, lanes 6 and 7). PhosphorImager analysis revealed that FAK6.7 exhibited 2–5-fold higher activity than wild-type FAK and that the activity of SuperFAK was 4–12 times greater than wild-type FAK. The immune complexes were also Western-blotted with a polyclonal antibody to FAK to demonstrate that equivalent levels of each FAK protein were present in the immunoprecipitates (Figure 2B, bottom panel). It can be concluded that the mutations introduced into SuperFAK and FAK6.7 lead to an increase in *in vitro* kinase activity compared with wild-type FAK.

Increased phosphorylation of downstream cellular proteins

Since SuperFAK and FAK6.7 had increased catalytic activity *in vitro*, the ability of these mutants to increase FAK signalling *in vivo* was investigated. Phosphotyrosine levels in CE cells expressing wild-type FAK or the FAK mutants were used as a measure of FAK signalling. Whole-cell lysates from CE cells were analysed by Western blotting with a phosphotyrosine

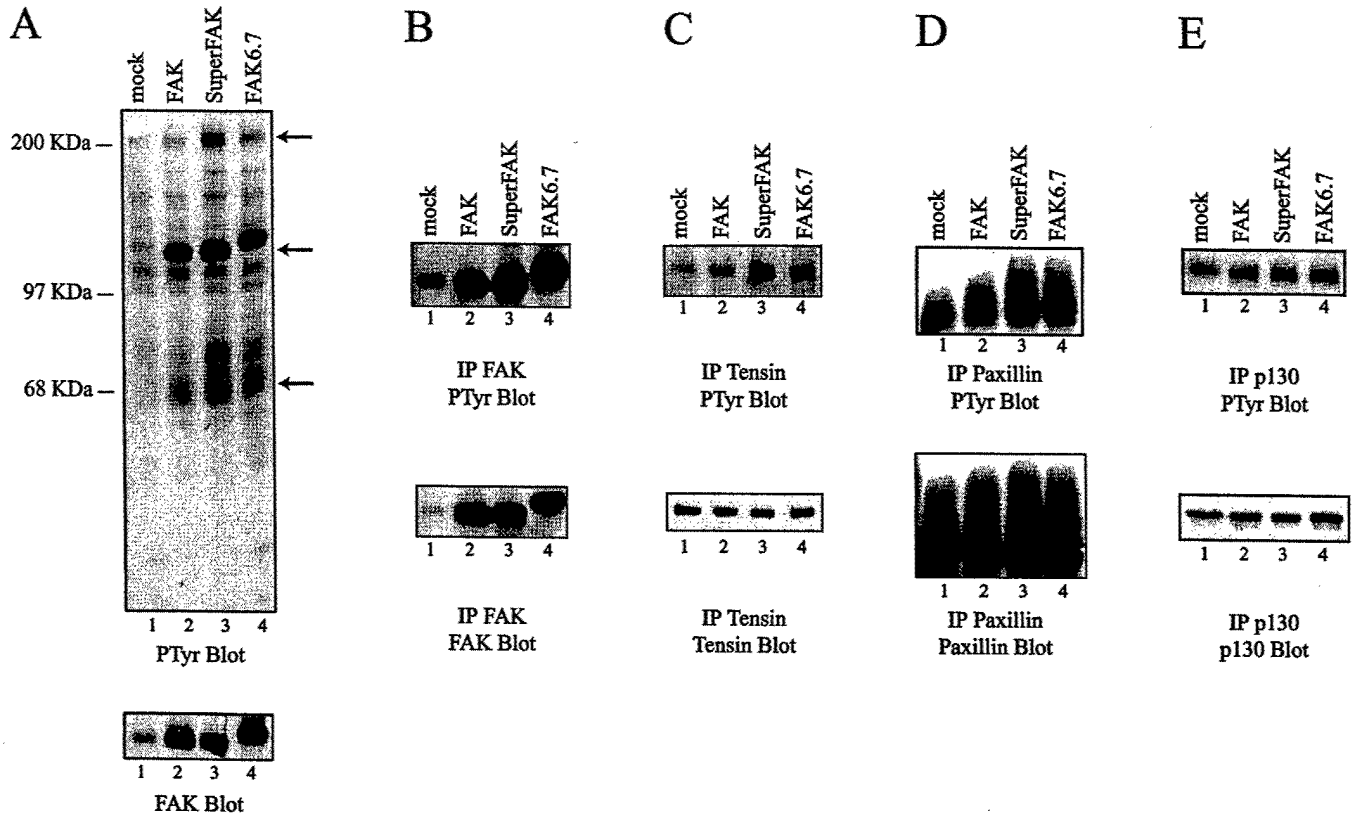

Figure 3 Elevated substrate phosphorylation in SuperFAK and FAK6.7 overexpressors

(**A**) Lysates (25 μg) of CE cells expressing empty vector (lane 1), FAK (lane 2), SuperFAK (lane 3) or FAK6.7 (lane 4) were Western-blotted with a phosphotyrosine antibody, RC20 (top panel). The positions of the molecular-mass markers are indicated on the left. The same lysates (25 μg) were Western-blotted with a polyclonal FAK antibody, BC4, to ensure equal expression of FAK protein (bottom panel). (**B**)–(**E**) FAK (**B**), tensin (**C**), paxillin (**D**) and p130^{cas} (**E**) were immunoprecipitated from CE cells (0.5–1 mg) expressing vector alone (lane 1), FAK (lane 2), SuperFAK (lane 3) or FAK6.7 (lane 4). The immune complexes were Western-blotted with a phosphotyrosine antibody, RC20 (**B**–**E**, top panels). The nitrocellulose membranes were stripped and re-probed for FAK, tensin, paxillin and p130^{cas} to ensure equal amounts of protein were being analysed (**B**–**E**, bottom panels).

antibody (Figure 3A). As previously described, overexpression of wild-type FAK leads to only a slight increase in cellular phosphotyrosine, and the major tyrosine phosphorylated band corresponds to FAK itself [45] (Figure 3A, top panel, lane 2). However, a striking increase in cellular phosphotyrosine was observed in SuperFAK overexpressors, and to a lesser extent in FAK6.7 overexpressors (Figure 3A, top panel, lanes 3 and 4). The major phosphotyrosine-containing proteins were approx. 200, 125 and 68–75 kDa in size (Figure 3A; arrows). On the basis of these molecular masses, and previous studies of FAK substrate phosphorylation [45], it seemed likely that tensin, FAK and paxillin are the major targets for enhanced phosphorylation in these cells.

In order to verify the identity of the proteins that were hyperphosphorylated, tensin, FAK, and paxillin were immunoprecipitated from lysates of CE cells overexpressing wild-type FAK or the mutant FAK proteins. The immune complexes were analysed by Western blotting for phosphotyrosine (Figures 3B–3D, top panels). FAK immunoprecipitated from CE cells transfected with wild-type FAK had a strong phosphotyrosine signal compared with mock-transfected cells owing to the expression of the exogenous wild-type FAK protein (Figure 3B, lane 2). A modest increase in the phosphorylation of the immunoprecipitated FAK was observed when SuperFAK, and, to a lesser extent, when FAK6.7 was expressed (Figure 3B, top panel, lanes 3 and 4). The phosphorylation of tensin and paxillin was slightly elevated upon expression of wild-type FAK compared with mock-transfected cells, as was previously described [45] (Figures 3C and 3D, top panels, lanes 2). The phosphotyrosine content of both tensin and paxillin was significantly increased in SuperFAK-expressing cells compared with wild-type-FAK-expressing cells (Figures 3C and 3D, top panels, lanes 3). Although not so dramatically, FAK6.7 also caused an elevation in the phosphotyrosine content of both tensin and paxillin compared with wild-type FAK (Figures 3C and 3D, top panels, lanes 4). The phosphotyrosine content of an additional FAK substrate, p130^{cas}, was similarly analysed. In contrast with the observed phosphorylation differences on tensin and paxillin, no significant change in the phosphotyrosine content of p130^{cas} was observed when either wild-type FAK, SuperFAK or FAK6.7 were expressed (Figure 3C, top panel). Control Western blots verified that equal amounts of protein were being immunoprecipitated in each case (Figures 3B–3E, bottom panels). These observations indicate that the elevated catalytic activity exhibited by SuperFAK and FAK6.7 is sufficient to amplify signalling events immediately downstream of FAK. Interestingly, phosphorylation of some FAK-associated tyrosine-phosphorylated proteins, i.e. paxillin, was increased, whereas phosphorylation

of others was not, i.e. p130^{cas}. Furthermore, the level of FAK substrate phosphorylation *in vivo* correlated with the catalytic activity of the kinases *in vitro*.

Cell-adhesion-dependent regulation of SuperFAK and FAK6.7 signalling

Cell adhesion is a major stimulus regulating FAK-mediated signal transduction. Upon cell detachment from the ECM, FAK becomes dephosphorylated, its catalytic activity declines and downstream signals are turned off [1]. To determine whether SuperFAK and FAK6.7 were constitutively active and able to send signals independent of cell adhesion, the cellular phosphotyrosine content was monitored in CE cells expressing wild-type FAK or the FAK mutants. The cells were either kept in culture or held in suspension for 45 min prior to lysis. Lysates were analysed by Western blotting with a phosphotyrosine antibody (Figure 4A, top panel). As described above (Figure 3A), expression of SuperFAK, and, to a lesser extent, FAK6.7, increased cellular phosphotyrosine to a higher level than wild-type FAK in cultured cells (Figure 4A, top panel, lanes 3, 5 and 7). When cells were detached, the cellular phosphotyrosine content was dramatically reduced in every cell type (Figure 4A, top panel, lanes 2, 4, 6 and 8). The level of phosphotyrosine in suspended SuperFAK and FAK6.7 cells was similar to the level of phosphotyrosine in suspended mock-transfected cells (Figure 4A, top panel, lanes 2, 6 and 8). Whole-cell lysates were also Western-blotted for FAK to ensure equal expression of FAK protein (Figure 4A, bottom panel). These observations indicate that the downstream phosphorylation signals mediated by SuperFAK and FAK6.7 are adhesion-dependent.

In vitro kinase assays were performed to investigate whether the decreased tyrosine phosphorylation observed upon loss of adhesion in SuperFAK and FAK6.7 overexpressors was due to a reduction in catalytic activity. FAK or the activated FAK mutants were immunoprecipitated from cultured CE cells or cells held in suspension. The immune complexes were incubated in kinase reaction buffer for 5 min at room temperature. The kinase reactions were stopped with the addition of Laemmli sample buffer and the samples analysed by SDS/PAGE and autoradiography. As expected from previous studies [1], the activity of wild-type FAK in suspended cells was decreased compared with the activity of FAK in adherent cells (Figure 4B, top panel, lanes 3 and 4). As shown above (Figure 2B), an elevation of the autophosphorylation activity of SuperFAK, and to a lesser extent FAK6.7, was observed in adherent cells (Figure 4B, top panel, lanes 3, 5 and 7). Although the kinase activity of SuperFAK and FAK6.7 decreased in the absence of an adhesion

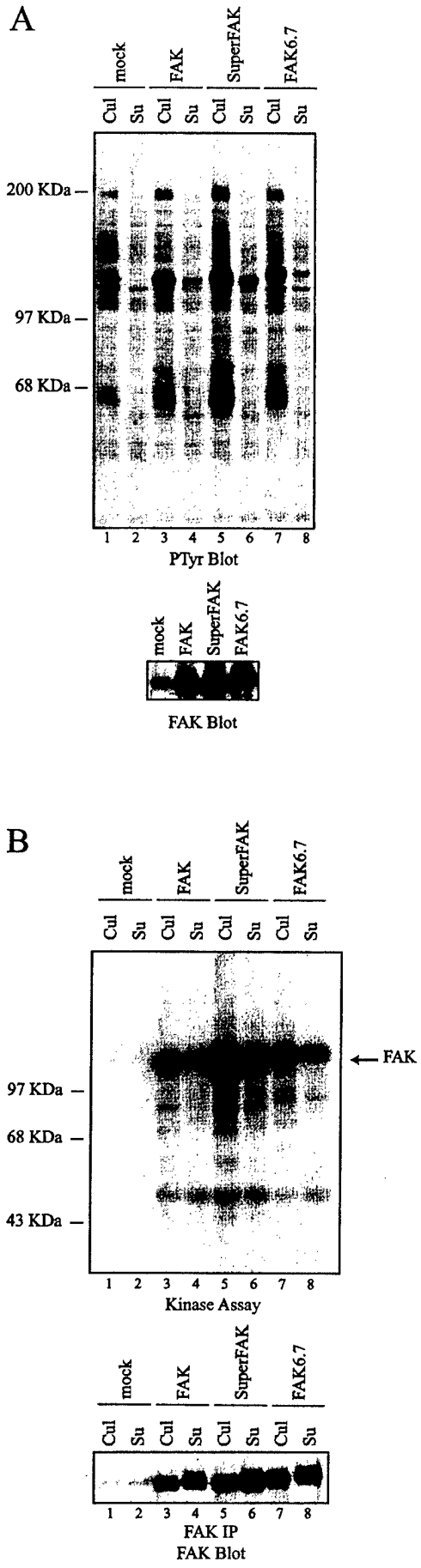

Figure 4 Adhesion-mediated regulation of FAK signalling and kinase activity

(**A**) The cellular phosphotyrosine content of CE cells expressing vector alone (lanes 1–2), FAK (lanes 3–4), SuperFAK (lanes 5–6) or FAK6.7 (lanes 7–8) was analysed in cells kept in culture (Cul; lanes 1, 3, 5 and 7) and in cells held in suspension for 45 min (Su; lanes 2, 4, 6 and 8). The lysates (25 μg) were Western-blotted using a phosphotyrosine antibody, RC20 (top panel). The same lysates (25 μg) were Western-blotted with a polyclonal FAK antibody, BC4, to ensure equal expression of FAK protein (bottom panel). (**B**) The kinase activity of the FAK mutants on loss of cell adhesion was analysed by using an immune-complex kinase assay. FAK was immunoprecipitated from CE cells (0.5–1 mg) expressing empty vector (lanes 1 and 2), FAK (lanes 3 and 4), SuperFAK (lanes 5 and 6) or FAK6.7 (lanes 7and 8) using a polyclonal FAK antibody, BC4. The immune complexes were incubated in kinase buffer containing [γ-^{32}P]ATP for 5 min. The samples were separated by SDS/8%-PAGE and ^{32}P incorporation was analysed by autoradiography (top panel). The immune complexes were also Western-blotted with a polyclonal FAK antibody, BC4, to ensure equal amounts of protein were being analysed (bottom panel). The positions of the molecular-mass markers are indicated on the left.

signal (Figure 4B, top panel, lanes 5 and 6, 7 and 8), SuperFAK and FAK6.7 still exhibited significantly higher catalytic activity compared with wild-type FAK (Figure 4B, top panels, lanes 4, 6 and 8). In fact, the autophosphorylation activity of SuperFAK from suspended cells was higher than the activity of wild-type FAK from adherent cells (Figure 4B, top panel, lanes 3 and 6). As a control for equal FAK loading, the immunoprecipitates were Western-blotted for FAK (Figure 4B, bottom panel). These results indicate that SuperFAK, and to a lesser extent FAK6.7, exhibit elevated catalytic activity in the presence or absence of adhesion. The loss of downstream phosphotyrosine signals in suspended cells can only be partially due to the decrease in catalytic activity of the FAK mutants, suggesting an additional mechanism(s) is involved in regulating tyrosine phosphorylation of focal adhesion-associated substrates.

In order to test whether the activated FAK mutants generated enhanced responses to physiological signals, tyrosine phosphorylation of paxillin was examined after cell adhesion to fibronectin. Paxillin was immunoprecipitated from lysates of cultured cells, cells kept in suspension for 45 minutes, or cells replated on fibronectin-coated dishes. The paxillin immune complexes were then Western-blotted with a phosphotyrosine antibody. As above (Figure 3D), SuperFAK, and to a lesser extent FAK6.7, increased the level of tyrosine phosphorylation on paxillin compared with wild-type FAK in cultured cells (Figure 5, top panel, lanes 4, 7 and 10). Upon loss of adhesion, paxillin phosphorylation was lost regardless of which FAK construct was expressed (Figure 5, top panel, compare Cul versus Su). This result correlates with the previous observation of phosphotyrosine levels in lysates of suspended cells (Figure 4A). Fibronectin-mediated adhesion induced tyrosine phosphorylation of paxillin in all the cells (Figure 5, top panel, lanes 3, 6, 9 and 12). The level of paxillin phosphorylation was significantly higher in cells expressing SuperFAK or FAK6.7 compared with cells expressing wild-type FAK (Figure 5, top panel, lanes 6, 9 and 12). The immune complexes were Western-blotted for paxillin in order to verify that equal amounts of protein were analysed (Figure 5, bottom panel). These observations demonstrate that the activated FAK mutants elevate FAK-mediated signals in response to a physiologically relevant stimulus.

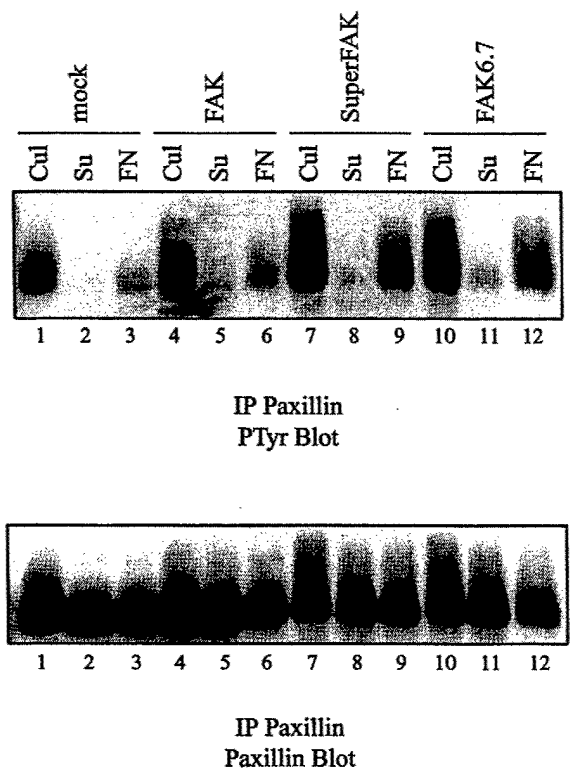

Figure 5 Physiological elevation of paxillin phosphorylation

The phosphorylation of the FAK substrate, paxillin, was analysed upon cell adhesion to fibronectin. Paxillin was immunoprecipitated from CE cells (0.5–1 mg) expressing vector alone (lanes 1–3), FAK (lanes 4–6), SuperFAK (lanes 7–9) or FAK6.7 (lanes 10–12). The cells were either kept in culture (Cul; lanes 1, 4, 7 and 10), held in suspension for 45 min (Su; lanes 2, 5, 8 and 11) or allowed to re-attach to fibronectin-coated dishes for 30 min (lanes 3, 6, 9 and 12). The paxillin immune complexes were Western-blotted with a phosphotyrosine antibody, RC20 (top panel). The nitrocellulose membranes were stripped and re-probed with a monoclonal paxillin antibody to ensure equal amounts of paxillin were being analysed (bottom panel).

Phosphorylation status of tyrosine residues in SuperFAK and FAK6.7

One possible mechanism through which the activated mutants might elevate downstream signalling is by recruiting Src family kinases into complex and/or enhancing signalling by Src family kinases. In order to investigate the role of Src in SuperFAK and FAK6.7 enhanced signalling, several lines of investigation were pursued. Lysates of CE cells expressing wild-type FAK, SuperFAK or FAK6.7 were Western-blotted with a phospho-specific antibody against Tyr^{397} (PY397), which is the Src SH2-domain-binding site on FAK (Figure 6A, top panel). To control for specificity, a FAK mutant with a phenylalanine-for-tyrosine substitution at this site was used as a negative control. PY397 recognized wild-type FAK but not the Y397F mutant (Figure 6A, lanes 2 and 3). Similar phosphorylation levels on Tyr^{397} were observed between wild-type FAK, SuperFAK and FAK6.7 (Figure 6A, top panel, lanes 2, 4 and 5). Lysates were Western-blotted for FAK to ensure equivalent amounts of FAK protein were analysed (Figure 6A, bottom panel). These observations indicate that neither SuperFAK nor FAK6.7 exhibit increased phosphorylation at Tyr^{397} *in vivo*.

To analyse the ability of the activated FAK mutants to associate with the Src SH2 domain *in vitro*, a GST fusion protein containing the SH2 domain of Src was used. GST fusion proteins were incubated with cell lysates and the bound proteins were Western-blotted to determine the amount of associated FAK. To ensure comparable levels of FAK protein expression, whole-cell lysates were Western-blotted for FAK (Figure 6B, bottom panel). GST alone was used to control for non-specific FAK binding (Figure 6B, top panel, lane 1). Similar amounts of exogenous wild-type FAK and the activated FAK mutants bound to GST–SrcSH2 (Figure 6B, top panel, lanes 3–5). These observations demonstrate that there were no changes in the ability of SuperFAK or FAK6.7 to associate with Src *in vitro* compared with wild-type FAK and are in agreement with the results of the PY397 Western blot (Figure 6A).

Co-immunoprecipitations were also performed to analyse the association of Fyn with FAK *in vivo*. Fyn was immunoprecipitated from CE cell lysates that overexpressed wild-type FAK, SuperFAK or FAK6.7. The immune complexes were Western-blotted for FAK (Figure 6C, top panel). The same amount of FAK, SuperFAK and FAK6.7 were co-immunoprecipitated with Fyn (Figure 6C, top panel, lanes 3–5). As a control, secondary antibody was used alone in an immunoprecipitation to demonstrate that the co-immunoprecipitation of FAK was specific (Figure 6C, top panel, lane 1). The blots were also stripped and reprobed for Fyn to ensure that equal amounts of Fyn were immunoprecipitated (Figure 6C, bottom

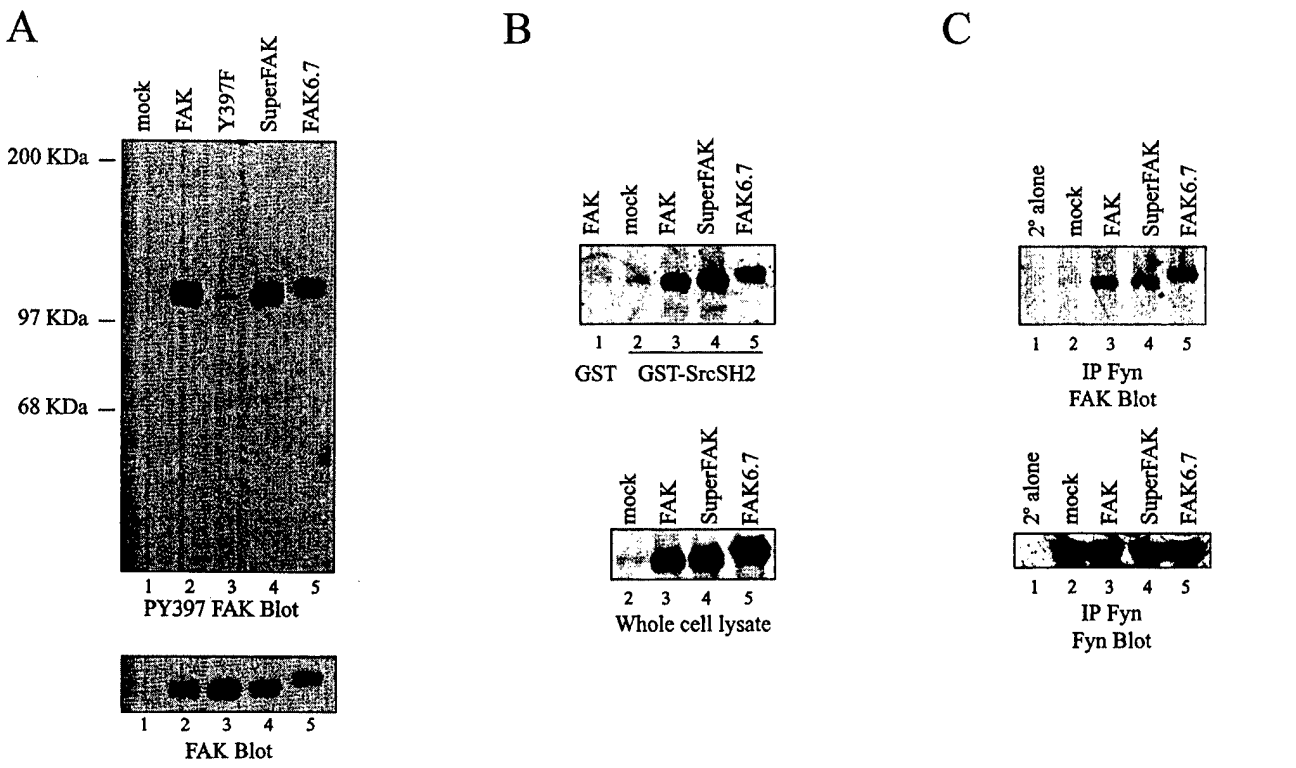

Figure 6 Phosphorylation of Tyr^{397} and FAK–Src complex formation

(**A**) The phosphorylation level of Tyr^{397} on FAK was analysed by Western blotting. Lysates (25 μg) from CE cells expressing vector alone (lane 1), FAK (lane 2), Y397F FAK (lane 3), SuperFAK (lane 4) or FAK6.7 (lane 5) were Western-blotted with a [PTyr^{397}]FAK phosphospecific polyclonal antibody ('Y397F'; top panel). Y397F FAK was used as a negative control for antibody specificity (lane 3). The nitrocellulose membrane was stripped and re-probed with a polyclonal FAK antibody, BC4, to ensure equal loading (bottom panel). (**B**) The association of the Src SH2 domain with FAK *in vitro* was analysed. CE cells (0.5 mg) expressing vector alone (lane 2), FAK (lanes 1, 3), SuperFAK (lane 4) or FAK6.7 (lane 5) were pre-cleared with GST (20 μg) for 1 h at 4 °C. The precleared lysates were then incubated with GST–SrcSH2 domain fusion protein (2 μg) for 2 h at 4 °C. GST alone (2 μg) was used to control for binding specificity (lane 1). Bound FAK was detected by Western blotting with a polyclonal FAK antibody, BC4 (top panel). To ensure equal expression of the FAK constructs, the same CE lysates (25 μg), expressing the empty vector (lane 2), FAK (lane 3), SuperFAK (lane 4), or FAK6.7 (lane 5) were Western-blotted with a polyclonal FAK antibody, BC4 (bottom panel). (**C**) The association between the FAK variants and Fyn *in vivo* was analysed by co-immunoprecipitation. Fyn was immunoprecipitated using a polyclonal Fyn antibody from CE cell lysates (0.5 mg) expressing vector alone (lane 2), FAK (lane 3), SuperFAK (lane 4), or FAK6.7 (lane 5). As a control for antibody specificity, the secondary antibody was used alone in an immunoprecipitation reaction with lysate of FAK expressing CE cells (lane 1). The immune complexes were Western-blotted with a polyclonal FAK antibody (top panel). The nitrocellulose membrane was stripped and re-probed with a polyclonal Fyn antibody to ensure equal amounts of Fyn were immunoprecipitated (bottom panel).

panel). These observations indicate that SuperFAK, FAK6.7 and wild-type FAK associate with equivalent amounts of Fyn *in vivo*.

Two approaches were taken to determine whether Src family kinases, in complex with FAK, were responsible for the enhanced catalytic activity of SuperFAK *in vitro*. First, kinase assays were performed in the presence of the Src inhibitor PP2. FAK was immunoprecipitated from CE lysates expressing vector alone, FAK or SuperFAK. The immune complexes were then incubated in kinase reaction buffer in the presence or absence of PP2 and terminated by the addition of Laemmli sample buffer. The samples were subject to SDS/PAGE and autoradiography. As described above (Figure 2B), SuperFAK had increased autophosphorylation levels compared with wild-type FAK (Figure 7A, lanes 3 and 5). Most importantly, the presence of PP2 had no effect on the autophosphorylation activity of wild-type FAK or SuperFAK (Figure 7A, lanes 3 and 4, and 5 and 6). In the second approach, an enolase substrate kinase assay was used to measure Src activity in FAK complexes. Immune complexes were incubated in reaction buffer with acid-denatured enolase and the samples analysed by SDS-PAGE and autoradiography. As a control, [Phe^{527}]Src immune complexes were used and shown to prominently phosphorylate enolase (Figure 7B, lanes 1 and 10). In the presence of 0.5 μM PP2, [Phe^{527}]Src autophosphorylation and enolase phosphorylation were significantly inhibited (results not shown and Figure 7, lanes 1 and 2, and 10 and 11). FAK immune complexes weakly phosphorylated enolase and SuperFAK immune complexes induced enhanced enolase phosphorylation (Figure 7B, lanes 6 and 8). However, the presence of PP2 had no effect on the phosphorylation of enolase by FAK or SuperFAK (Figure 7B, lanes 6 and 7, and 8 and 9). This observation suggests that the weak phosphorylation of enolase by FAK and SuperFAK immune complexes was due to FAK activity and not due to co-immunoprecipitating Src family kinase activity. These results demonstrate that the increased activity of SuperFAK *in vitro* is not due to enhanced activity of co-immunoprecipitating Src kinases.

The results of the phosphospecific-antibody Western blots, *in vitro* binding assays, Fyn co-immunoprecipitations and *in vitro* kinase assays indicate that FAK, SuperFAK and FAK6.7 are equally phosphorylated on Tyr^{397} and bind comparable amounts of Src kinases. However, there remains the possibility that these FAK variants might elevate Src activity *in vivo*. In order to investigate this possibility, the activation state of Src was examined using phosphospecific antibodies. Cell lysates were Western-blotted with phosphospecific antibodies to Tyr^{416}

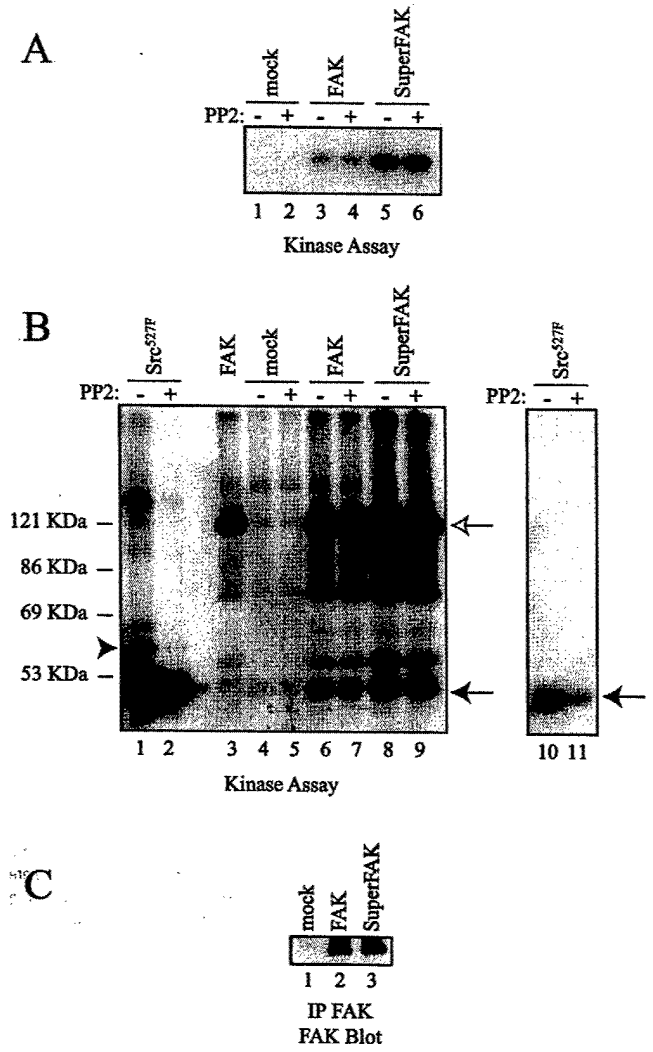

Figure 7 Effect of Src inhibition on FAK kinase activity *in vitro*

The effects of PP2, a pharmacological inhibitor of Src, on FAK kinase activity was monitored using an immune complex kinase assay. (**A**) FAK was immunoprecipitated from CE lysates (1 mg) expressing empty vector (lanes 1 and 2), FAK (lanes 3 and 4), or SuperFAK (lanes 5 and 6), using a polyclonal FAK antibody, BC4. The immune complexes were incubated in kinase buffer containing [γ-^{32}P]ATP for 8 min in the presence (+) of 0.5 μM PP2 or vehicle alone (DMSO; —). (**B**) Src was immunoprecipitated from CE lysates (1 mg) expressing [Phe^{527}]Src ('Src^{527F}'), using a monoclonal Src antibody, EC10. The Src (lanes 1 and 2) and FAK (lanes 4–10) immune complexes (isolated as described in **A**) were incubated with 5 μg of acid-denatured enolase in enolase kinase buffer containing [γ-^{32}P]ATP in the presence (+) of 0.5 μM PP2 or vehicle alone (DMSO; —) for 13 min. As a control, a FAK immune complex was incubated in an autophosphorylation reaction in the absence of enolase (lane 3). All kinase reaction samples were subjected to SDS/PAGE and autoradiography. The position of Src (closed arrowhead), FAK (open arrow) and enolase (closed arrow) are indicated. An underexposure of lanes 1 and 2 is shown in lanes 10 and 11. The positions of the molecular-mass markers are indicated on the left. (**C**) The FAK immune complexes were also Western-blotted using a polyclonal FAK antibody, BC4, to ensure equal amounts of protein were being analysed.

(PY416), which is in the activation loop of Src, or to Tyr^{527} (PY527), which is a negative regulatory site of phosphorylation. As controls for specificity, CE cells expressing c-Src or [Phe^{527}]-Src were analysed. Under steady-state conditions, c-Src is largely inactive, and phosphorylation of Tyr^{416} is low, whereas phosphorylation of Tyr^{527} is high. [Phe^{527}]Src cannot be phosphorylated on Tyr^{527}, rendering it highly active and resulting in high phosphorylation of Tyr^{416}. The phosphospecific PY416 antibody prominently recognized [Phe^{527F}]Src and weakly recognized c-Src, suggesting that the antibody was specific to Tyr^{416} (Figure 8A, top panel). The specificity of PY527 was also verified, since this antibody recognized c-Src, but not the Y527F Src mutant (Figure 8A, middle panel). Upon co-expression with wild-type FAK, an increase in Tyr^{416} phosphorylation was observed on c-Src (Figure 8B, top panel, lanes 1 and 2), indicating that co-expression with FAK can activate Src. The level of Tyr^{416} phosphorylation when SuperFAK or FAK6.7 was co-expressed with c-Src was the same as in cells co-expressing wild-type FAK and c-Src (Figure 8B, top panel, lanes 2–4). The level of phosphorylation of Tyr^{527} on c-Src remained unchanged, regardless of co-expression of FAK proteins (Figure 8B, second panel). To monitor the amounts of protein being analysed, Src and FAK Western blots were performed (Figure 8A, bottom panel, and Figure 8B, third and bottom panels). The observations indicate that wild-type FAK, SuperFAK and FAK6.7 have similar effects on the phosphorylation of Src. Therefore the mechanism by which SuperFAK and FAK6.7 send amplified signals *in vivo* is not via enhanced activation of Src kinases.

The phosphorylation of Tyr^{925} in the C-terminus of FAK creates a binding site for the SH2 domain of Grb2 ([11,12], but see [12a]). To explore phosphorylation of Tyr^{925} a phospho-specific antibody was initially used. However, under the blotting conditions used, this antibody recognized a FAK mutant with a phenylalanine substitution for Tyr^{925} (results not shown), precluding its use in this analysis. As an alternative approach, Tyr^{925} phosphorylation in SuperFAK and FAK6.7 was investigated by examining the ability of the FAK mutants to associate with the Grb2SH2 domain *in vitro*. A GST fusion protein containing the Grb2SH2 domain was incubated with vanadate-treated lysates of CE cells expressing FAK, or the activated FAK variants, and bound proteins analysed by Western blotting for FAK. To ensure comparable levels of FAK protein expression, whole cell lysates were Western-blotted for FAK (Figure 9, bottom panel). FAK bound to the GST–GrbSH2 domain, but failed to associate with GST alone (Figure 9, top panel, lanes 1 and 3). Higher levels of SuperFAK were found associated with the Grb2 SH2 domain compared with wild-type FAK (Figure 9, top panel, lanes 3 and 4). In contrast, similar amounts of FAK6.7 and wild-type FAK associated with the Grb2 SH2 domain (Figure 9, top panel, lanes 3 and 5). These results suggest that SuperFAK has elevated phosphorylation at Tyr^{925}.

Increased motility of T47D cells expressing SuperFAK

Since SuperFAK and FAK6.7 increased FAK signalling biochemically, the ability of these mutants to impinge upon FAK-mediated biological processes was tested. In order to investigate the effects of the activated FAK proteins on cell motility, T47D cells, a breast-cancer epithelial cell line, were utilized. The T47D cells were engineered to stably express the receptor for the avian subgroup A retrovirus, Tva800 [41]. Whereas, the parental T47D cells are resistant to infection with avian retroviruses since the cells lack the viral receptor, the derived cell line, T47D/Tva, is susceptible to infection with avian A type retroviruses. The FAK constructs, cloned into RCAS type A, were transfected into CE cells. Virus produced in CE cells was collected and used to infect the T47D/Tva cells to generate populations of cells expressing each of the FAK proteins of interest. Western blots were performed to examine expression of FAK proteins following infection of the T47D/Tva cells. These results demonstrated that FAK, SuperFAK and FAK6.7 were expressed to high levels in the T47D/Tva cells (Figure 10A).

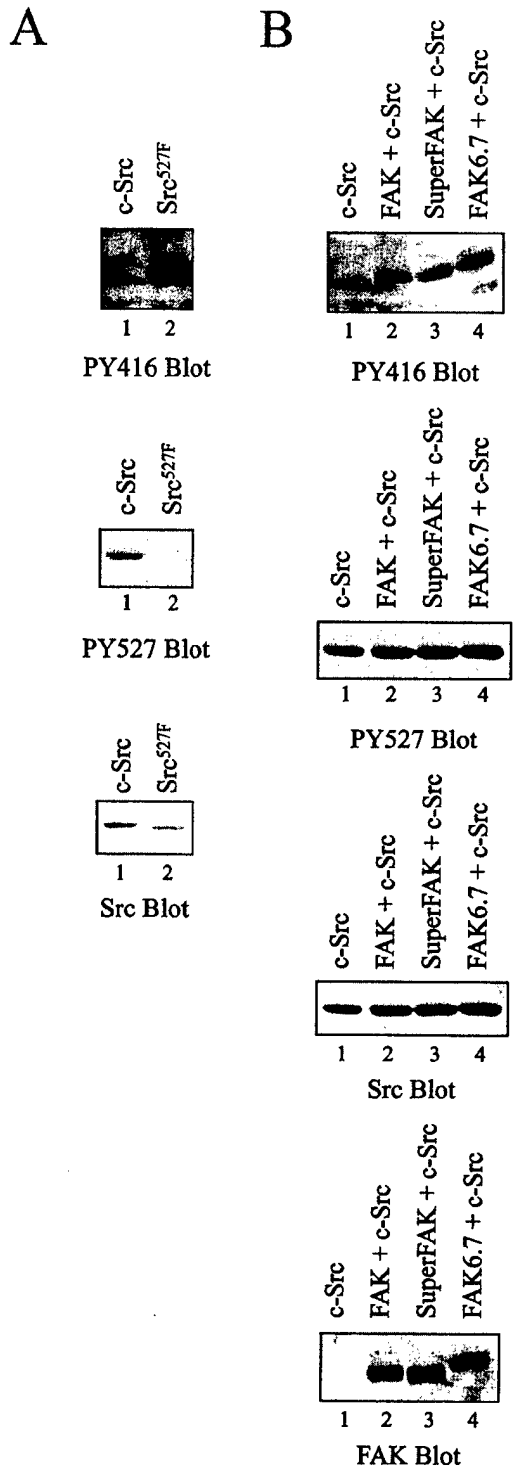

Figure 8 Phosphorylation status of Src in cells co-expressing FAK and Src

(**A**) The phosphorylation status of the activation loop tyrosine (Tyr^{416}) and the inhibitory tail tyrosine (Tyr^{527}) in Src were analysed by Western blotting. In order to ensure antibody specificity, CE cell lysates (25 μg) overexpressing c-Src (lane 1) or Src with a tyrosine-527-to-phenylalanine substitution, [Phe^{527}]Src ('Src^{527F}'), (lane 2) were Western-blotted with polyclonal Src phosphospecific antibodies against phosphorylated Tyr^{416} ('PY416'; top panel) or phosphorylated Tyr^{527} ('PY527'; middle panel). The nitrocellulose membrane was stripped and re-probed with a monoclonal Src antibody, EC10, to ensure equal expression (bottom panel). (**B**) CE cell lysates (25 μg) co-expressing c-Src and vector alone (lane 1), FAK (lane 2), SuperFAK (lane 3) or FAK6.7 (lane 4) were Western-blotted with a Src activation-loop phosphospecific tyrosine antibody, PY416 (top panel), or a Src inhibitory tail phosphospecific tyrosine antibody, PY527 (second panel). The nitrocellulose membranes were stripped and re-probed with a monoclonal Src antibody, EC10 (third panel), or a polyclonal FAK antibody, BC4 (bottom panel), to ensure equal amounts of protein were being analysed.

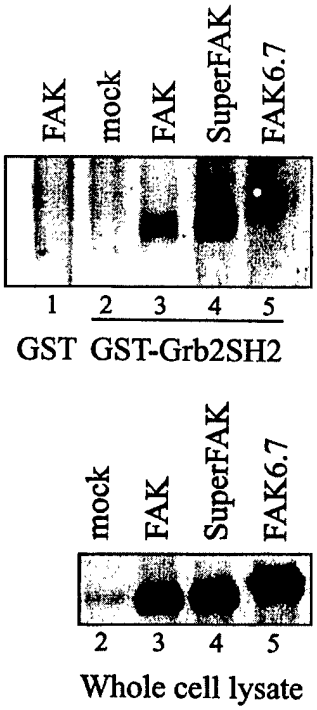

Figure 9 Phosphorylation of Tyr^{925} is increased in cells expressing SuperFAK

The phosphorylation status of Tyr^{925} on FAK was analysed using an *in vitro* binding assay. CE cells expressing empty vector (lane 2), FAK (lanes 1, 3), SuperFAK (lane 4) or FAK6.7 (lane 5) were treated overnight with vanadate (50 μM), then lysed. The CE lysates (1 mg) were precleared with GST (20 μg) for 1 h at 4 °C. Pre-cleared lysates were then incubated with a GST–Grb2SH2 fusion protein (lanes 2–5) or GST alone (lane 1). Bound FAK was detected by Western blotting with a polyclonal FAK antibody, BC4 (top panel). The same lysates (25 μg) were Western-blotted with a polyclonal FAK antibody, BC4, to ensure equal expression of the FAK constructs (bottom panel).

Using a transwell motility assay as previously described [44], the haptotactic motility of the cells was analysed. Populations of infected T47D/Tva cells were allowed to migrate for 20–22 h through a porous transwell membrane coated on the underside with collagen I (40 μg/ml). The non-motile cells were removed from the top of the membrane, and the cells that migrated to the underside of the membrane were stained and counted. The average fold change in motility was plotted (Figure 10B). The motility of T47D cells was significantly increased by FAK overexpression, which is consistent with previous findings demonstrating a role for FAK in regulating motility in other cell types [16,18,19]. Expression of SuperFAK further increased the motility of T47D cells compared with cells expressing FAK, whereas FAK6.7 was less efficient than wild-type FAK in enhancing haptotaxis. These observations demonstrate the ability of SuperFAK to amplify a FAK-mediated biological response.

DISCUSSION

In the present study we describe the construction of two activated mutants of FAK: SuperFAK and FAK6.7. SuperFAK, and to a lesser extent FAK6.7, showed increased catalytic activity *in vitro* compared with wild-type FAK. The catalytic activity of both

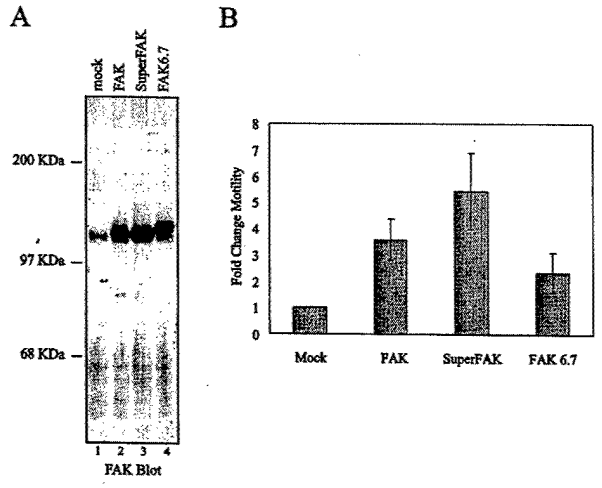

Figure 10 Elevation of T47D/Tva motility in cells expressing FAK or SuperFAK

(**A**) The expression of the FAK proteins in T47D/Tva cells 10 days after infection was analysed by Western blotting. Lysates (25 μg) of T47D/Tva cells infected with the empty retroviral vector (lane 1) or retrovirus containing the FAK (lane 2), SuperFAK (lane 3), or FAK6.7 (lane 4) cDNAs were Western-blotted with a polyclonal FAK antibody, BC4. The positions of the molecular-mass markers are indicated on the left. (**B**) The motility of T47D/Tva cells expressing empty vector (mock), FAK, SuperFAK or FAK6.7 was measured in a transwell system. Cells were allowed to migrate to the underside of a collagen-coated transwell membrane for 20–22 h. The number of cells that reached the underside of the membrane were counted. The average ($\pm$S.E.M.) fold change in migration from 11 experiments is shown. The difference in motility between FAK and mock, and between SuperFAK and mock were statistically significant ($P < 0.05$).

mutants was partially regulated by adhesion, since both exhibited reduced catalytic activity from cells in suspension. Nevertheless, the enzymic activity of SuperFAK from suspended cells greatly exceeded the activity of wild-type FAK. The elevated activity of SuperFAK and FAK6.7 translated into increased downstream biochemical signals *in vivo*, as demonstrated by the phosphorylation of FAK substrates, i.e. paxillin and tensin. Despite the high level of catalytic activity of SuperFAK in suspended cells, phosphorylation of downstream substrates by SuperFAK, as well as FAK6.7, was dependent on adhesion. The expression of the activated FAK mutants in CE cells had no effect on cell morphology, and their subcellular localization was identical with that of wild-type FAK (results not shown). Furthermore, the elevation of the FAK signal upon SuperFAK expression leads to increased motility of breast-cancer cells. Thus the activated mutant of FAK, SuperFAK, may be a powerful tool that can be used to study the consequences of increased and/or aberrant FAK signalling in a variety of physiological contexts.

In many protein kinases, phosphorylation of residues in the activation loop, analogous to Tyr^{576} and Tyr^{577} in FAK, is a mechanism of enzyme activation [46]. The negative charge introduced by the phosphate groups stabilizes the active conformation, in which the ATP and the substrate-binding sites of the kinase domain are accessible [46]. In the case of the Y576E/Y577E FAK mutant, no change in catalytic activity or downstream tyrosine phosphorylation signals was observed. It was expected that the introduction of the charged residues might mimic phosphorylation of the tyrosine residues, which normally enhances the catalytic activity of FAK [3]. However, it appears that the tyrosine-to-glutamic acid substitutions in Y576E/Y577E were not sufficient to cause the conformational change necessary for the activation of FAK. Interestingly, although substitutions of acidic residues for activation loop phosphorylation sites can activate some protein kinases, e.g. MAPK/extracellular-signal-related kinase (ERK) kinase ('MEK') [46], there are no reports of mutational activation of tyrosine kinases using this strategy. In the case of SuperFAK, the double substitution of the activation-loop lysine residues (Lys^{578} and Lys^{581}) with glutamic acid residues did lead to activation of the kinase, whereby catalytic activity was elevated and downstream biochemical and cellular events were augmented. Thus the substitution of acidic residues for basic residues in the activation loop results in catalytic activation, as originally reported for FGFR3 [35]. The negatively charged glutamic acid residues may alter the conformation in such a way as to mimic the conformational change that occurs when the regulatory tyrosine residues in the activation loop of FAK are phosphorylated. Alternatively, the activation of SuperFAK could be explained by an increase in the level of phosphorylation of the regulatory tyrosine residues in the kinase domain of FAK, Tyr^{576} and Tyr^{577}. The double lysine- to-glutamic acid substitution may alter the recognition of FAK by either a tyrosine kinase, likely Src, or a tyrosine phosphatase resulting in elevated phosphorylation of Tyr^{576} and Tyr^{577}, leading to enhanced activity.

FAK6.7 is an avian version of an alternatively spliced variant of FAK found in rat brain [36]. As previously reported, FAK6.7 has elevated autophosphorylation activity *in vitro* [36,48]. In addition, we have shown that this mutant can also elevate tyrosine phosphorylation of substrates *in vivo*. The mechanism of activation of the mutant is not clear. It has been speculated that alterations around the autophosphorylation site might alter the level of phosphorylation at that site. It has been reported that FAK6.7 expressed in COS-7 cells does exhibit elevated phosphorylation at Tyr^{397} [48]. However, our analysis in fibroblasts does not show an elevation of phosphorylation at Tyr^{397} *in vivo*. Increased tyrosine phosphorylation of FAK at Tyr^{397} could result in increased recruitment of Src, but our results suggest that FAK6.7 does not exhibit increased Src binding *in vivo*. The discrepancy between published results and the results obtained in the present study may be due to the different cell types and expression systems used or the use of different Tyr^{397} phospho-specific antibodies. In the light of our observations, the mechanism of activation of FAK6.7 still remains to be solved.

Src plays an important role in biochemical signalling via FAK. Src can bind autophosphorylated FAK and phosphorylate activation-loop residues to further promote the activity of FAK [3]. In addition, recruitment of Src into a complex with FAK may direct phosphorylation of paxillin and tensin *in vivo* by Src [45]. These observations raise the question of the role Src plays in signalling by SuperFAK and FAK6.7. Experiments using phosphospecific antibodies and GST fusion proteins indicate that there was no significant change in the level of phosphorylation of Tyr^{397} of the activated FAK mutants compared with wild-type FAK. Further co-immunoprecipitation experiments reveal no difference in the association of Fyn with wild-type FAK or the activated mutants. Similarly, experiments using a Src inhibitor in FAK kinase assays also suggest that the increase in catalytic activity of SuperFAK is not due to increased association with Src kinases. These observations suggest that enhanced signalling by SuperFAK and FAK6.7 is not due to increased recruitment of Src kinases. A FAK-dependent mechanism of Src activation has been proposed [7,10]. However, experiments using Src phospho-specific antibodies demonstrate that SuperFAK and FAK6.7 activate Src *in vivo* to the same extent as wild-type FAK. Thus the enhanced effects of SuperFAK and FAK6.7 on downstream signalling might be independent of Src. Furthermore, from these studies it seems likely that paxillin and tensin are serving as direct substrates for SuperFAK and FAK6.7. In contrast, expres-

sion of SuperFAK or FAK6.7 did not result in elevated tyrosine phosphorylation of p130^{cas}. This observation is consistent with the hypothesis that Src is the major kinase responsible for tyrosine phosphorylation of p130^{cas} [49,50].

One interesting observation was the apparent increase in phosphorylation of Tyr^{925} in SuperFAK relative to wild-type FAK and FAK6.7. Src has been shown to be the kinase responsible for phosphorylation of Tyr^{925} ([12], but see [12a]). However, since there is no difference in the association of FAK and SuperFAK with Src family kinases, the mechanism leading to phosphorylation of this site is unclear. Since phosphorylation of Tyr^{925} creates a Grb2-binding site linking FAK with the MAPK cascade [51] and SuperFAK exhibits elevated levels of phosphorylation at Tyr^{925}, it was anticipated that SuperFAK might induce enhanced activation of ERK. However, no significant effect of SuperFAK upon adhesion-mediated or serum-mediated ERK activation was observed in CE cells (results not shown).

In our studies, the catalytic activity of SuperFAK and FAK6.7 was reduced when cells were held in suspension. However, in the absence of an adhesion signal, SuperFAK and FAK6.7 still displayed much higher kinase activity *in vitro* compared with wild-type FAK. Thus the kinase activity of SuperFAK and FAK6.7 is only partially regulated by adhesion. These observations suggest that the mutants may not be constitutively active, since they still require a cell-adhesion stimulus for maximal activation. Nevertheless, these mutants exhibit higher enzymic activity compared with wild-type FAK. Although the catalytic activity of SuperFAK and FAK6.7 remained high in the absence of an adhesion signal, tyrosine phosphorylation of downstream effectors did not occur in cells in suspension. These observations indicate that the high catalytic activity of SuperFAK and FAK6.7 is insufficient to induce substrate phosphorylation in suspended cells. There are a number of possible explanations for this observation. The absence of FAK substrate phosphorylation could be attributed to the action of cellular phosphatases, which are more active, or have increased access to substrates, when cells are in suspension. Alternatively, the proximity of the activated FAK mutants to its substrates represents another potential method of regulation. The assembly of proteins into focal adhesions may be required not only for the efficient activation of FAK, but also for the clustering of FAK with its substrates to promote their phosphorylation and the transmission of FAK downstream signals.

Several FAK-binding partners have been implicated in mediating FAK-dependent cell motility, including Src family kinases and PI 3-kinase [6,19]. Src kinases are likely to phosphorylate downstream substrates to regulate motility, and one important substrate implicated in cell motility is p130^{cas} [20,52]. A FAK mutant that cannot bind p130^{cas} or induce its tyrosine phosphorylation is defective for induction of motility, implicating p130^{cas} in the regulation of motility by FAK [20]. It may therefore be noteworthy that the FAK and SuperFAK do not induce p130^{cas} tyrosine phosphorylation, yet do promote cell motility. FAK may utilize different signalling pathways to control cell motility in different cell types. Further experimentation is required to test this hypothesis.

The characterization of SuperFAK and FAK6.7 has demonstrated the ability of these constructs to increase FAK-mediated signals. Other membrane-bound constitutively activated FAK constructs, CD2FAK and myrFAK, have been described [28,29]. The best characterized of these FAK variants, CD2FAK, exhibits biochemical properties different from those of SuperFAK. First, its expression does not promote dramatic increases of tyrosine phosphorylation of substrates in adherent cells [23,50]. Secondly, in suspended cells expressing CD2FAK, the tyrosine phosphorylation of paxillin and p130^{cas} are sustained [23,50]. Presumably, constitutive CD2FAK and MyrFAK signalling can be explained by their constitutive association with the membrane and membrane-bound proteins such as Src. This contrasts with the hyperactive signalling generated by SuperFAK and FAK6.7 in response to normal physiological stimuli. In this regard, SuperFAK may better mimic pathological situations exhibiting overexpression of FAK. In different scenarios, the membrane-bound chimaeras and SuperFAK may be utilized to study the role of aberrant FAK signalling, alone or in combination with other signalling partners, in the development of human disease.

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REFERENCES

1 Schaller, M. D. (2001) Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim. Biophys. Acta **1540**, 1–21
2 Hildebrand, J. D., Schaller, M. D. and Parsons, J. T. (1993) Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. J. Cell Biol. **123**, 993–1005
3 Calalb, M. B., Polte, T. R. and Hanks, S. K. (1995) Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. Mol. Cell Biol. **15**, 954–963
4 Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R. and Parsons, J. T. (1994) Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. Mol. Cell Biol. **14**, 1680–1688
5 Han, D. C. and Guan, J. L. (1999) Association of focal adhesion kinase with Grb7 and its role in cell migration. J. Biol. Chem. **274**, 24425–24430
6 Reiske, H. R., Kao, S. C., Cary, L. A., Guan, J. L., Lai, J. F. and Chen, H. C. (1999) Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. J. Biol. Chem. **274**, 12361–12366
7 Schlaepfer, D. D. and Hunter, T. (1997) Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. J. Biol. Chem. **272**, 13189–13195
8 Zhang, X., Chattopadhyay, A., Ji, Q. S., Owen, J. D., Ruest, P. J., Carpenter, G. and Hanks, S. K. (1999) Focal adhesion kinase promotes phospholipase C-γ1 activity. Proc. Natl. Acad. Sci. U.S.A. **96**, 9021–9026
9 Fincham, V. J., Brunton, V. G. and Frame, M. C. (2000) The SH3 domain directs acto-myosin-dependent targeting of v-Src to focal adhesions via phosphatidylinositol 3-kinase. Mol. Cell Biol. **20**, 6518–6536
10 Thomas, J. W., Ellis, B., Boerner, R. J., Knight, W. B., White, G. C. and Schaller, M. D. (1998) SH2- and SH3-mediated interactions between focal adhesion kinase and Src. J. Biol. Chem. **273**, 577–583
11 Schlaepfer, D. D., Hanks, S. K., Hunter, T. and van der Geer, P. (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature (London) **372**, 786–791
12 Schlaepfer, D. D. and Hunter, T. (1996) Evidence for *in vivo* phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. Mol. Cell Biol. **16**, 5623–5633
12a Erratum (1996) Mol. Cell. Biol. **16**, 7182–7184
13 Harte, M. T., Hildebrand, J. D., Burnham, M. R., Bouton, A. H. and Parsons, J. T. (1996) p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. J. Biol. Chem. **271**, 13649–13655
14 Polte, T. R. and Hanks, S. K. (1995) Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. Proc. Natl. Acad. Sci. U.S.A. **92**, 10678–10682
15 Fincham, V. J., Wyke, J. A. and Frame, M. C. (1995) v-Src-induced degradation of focal adhesion kinase during morphological transformation of chicken embryo fibroblasts. Oncogene **10**, 2247–2252
15a Erratum (1995) Oncogene **11**, 2185

16 Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M. and Yamamoto, T. (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature (London) **377**, 539–544
17 Ren, X. D., Kiosses, W. B., Sieg, D. J., Otey, C. A., Schlaepfer, D. D. and Schwartz, M. A. (2000) Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. J. Cell Sci. **113**, 3673–3678
18 Gilmore, A. P. and Romer, L. H. (1996) Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. Mol. Biol. Cell **7**, 1209–1224
19 Cary, L. A., Chang, J. F. and Guan, J. L. (1996) Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. J. Cell Sci. **109**, 1787–1794
20 Cary, L. A., Han, D. C., Polte, T. R., Hanks, S. K. and Guan, J. L. (1998) Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. J. Cell Biol. **140**, 211–221
21 Hungerford, J. E., Compton, M. T., Matter, M. L., Hoffstrom, B. G. and Otey, C. A. (1996) Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. J. Cell Biol. **135**, 1383–1390
22 Xu, L. H., Owens, L. V., Sturge, G. C., Yang, X., Liu, E. T., Craven, R. J. and Cance, W. G. (1996) Attenuation of the expression of the focal adhesion kinase induces apoptosis in tumor cells. Cell Growth Differ. **7**, 413–418
23 Frisch, S. M., Vuori, K., Ruoslahti, E. and Chan-Hui, P. Y. (1996) Control of adhesion-dependent cell survival by focal adhesion kinase. J. Cell Biol. **134**, 793–799
24 Chan, P. C., Lai, J. F., Cheng, C. H., Tang, M. J., Chiu, C. C. and Chen, H. C. (1999) Suppression of ultraviolet irradiation-induced apoptosis by overexpression of focal adhesion kinase in Madin–Darby canine kidney cells. J. Biol. Chem. **274**, 26901–26906
25 Sonoda, Y., Matsumoto, Y., Funakoshi, M., Yamamoto, D., Hanks, S. K. and Kasahara, T. (2000) Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. J. Biol. Chem. **275**, 16309–16315
26 Almeida, E. A., Ilic, D., Han, Q., Hauck, C. R., Jin, F., Kawakatsu, H., Schlaepfer, D. D. and Damsky, C. H. (2000) Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH_2-terminal kinase. J. Cell Biol. **149**, 741–754
27 Zhao, J. H., Reiske, H. and Guan, J. L. (1998) Regulation of the cell cycle by focal adhesion kinase. J. Cell Biol. **143**, 1997–2008
28 Chan, P. Y., Kanner, S. B., Whitney, G. and Aruffo, A. (1994) A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK. J. Biol. Chem. **269**, 20567–20574
29 Igishi, T., Fukuhara, S., Patel, V., Katz, B. Z., Yamada, K. M. and Gutkind, J. S. (1999) Divergent signaling pathways link focal adhesion kinase to mitogen-activated protein kinase cascades. Evidence for a role of paxillin in c-Jun NH_2-terminal kinase activation. J. Biol. Chem. **274**, 30738–30746
30 Shu, H. K., Pelley, R. J. and Kung, H. J. (1990) Tissue-specific transformation by epidermal growth factor receptor: a single point mutation within the ATP-binding pocket of the erbB product increases its intrinsic kinase activity and activates its sarcomagenic potential. Proc. Natl. Acad. Sci. U.S.A. **87**, 9103–9107
31 Hofstra, R. M., Landsvater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J. W., van Amstel, H. K. and Romeo, G. (1994) A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. Nature (London) **367**, 375–376
32 Nagata, H., Worobec, A. S., Oh, C. K., Chowdhury, B. A., Tannenbaum, S., Suzuki, Y. and Metcalfe, D. D. (1995) Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. Proc. Natl. Acad. Sci. U.S.A. **92**, 10560–10564
33 Park, W. S., Dong, S. M., Kim, S. Y., Na, E. Y., Shin, M. S., Pi, J. H., Kim, B. J., Bae, J. H., Hong, Y. K., Lee, K. S. et al. (1999) Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. Cancer Res. **59**, 307–310
34 Santoro, M. M., Penengo, L., Minetto, M., Orecchia, S., Cilli, M. and Gaudino, G. (1998) Point mutations in the tyrosine kinase domain release the oncogenic and metastatic potential of the Ron receptor. Oncogene **17**, 741–749
35 Webster, M. K., D'Avis, P. Y., Robertson, S. C. and Donoghue, D. J. (1996) Profound ligand-independent kinase activation of fibroblast growth factor receptor 3 by the activation loop mutation responsible for a lethal skeletal dysplasia, thanatophoric dysplasia type II. Mol. Cell Biol. **16**, 4081–4087
36 Burgaya, F., Toutant, M., Studler, J. M., Costa, A., Le Bert, M., Gelman, M. and Girault, J. A. (1997) Alternatively spliced focal adhesion kinase in rat brain with increased autophosphorylation activity. J. Biol. Chem. **272**, 28720–28725
37 Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B. and Parsons, J. T. (1992) pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc. Natl. Acad. Sci. U.S.A. **89**, 5192–5196
38 Schaller, M. D., Borgman, C. A. and Parsons, J. T. (1993) Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125FAK. Mol. Cell Biol. **13**, 785–791
39 Schaller, M. D., Hildebrand, J. D. and Parsons, J. T. (1999) Complex formation with focal adhesion kinase: a mechanism to regulate activity and subcellular localization of Src kinases. Mol. Biol. Cell **10**, 3489–3505
40 Reynolds, A. B., Roesel, D. J., Kanner, S. B. and Parsons, J. T. (1989) Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cells expressing oncogenic variants of the avian cellular src gene. Mol. Cell Biol. **9**, 629–638
41 Young, J. A., Bates, P. and Varmus, H. E. (1993) Isolation of a chicken gene that confers susceptibility to infection by subgroup A avian leukosis and sarcoma viruses. J. Virol. **67**, 1811–1816
41a Shen, Y. and Schaller, M. D. (1999) Focal adhesion targeting: the critical determinant of FAK regulation and substrate phosphorylation. Mol. Biol. Cell **10**, 2507–2518
42 Shen, Y., Schneider, G., Cloutier, J. F., Veillette, A. and Schaller, M. D. (1998) Direct association of protein-tyrosine phosphatase PTP-PEST with paxillin. J. Biol. Chem. **273**, 6474–6481
43 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
43a Smith, D. B. and Johnson, K. S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67**, 31–40
44 Keely, P. J., Fong, A. M., Zutter, M. M. and Santoro, S. A. (1995) Alteration of collagen-dependent adhesion, motility and morphogenesis by the expression of antisense alpha 2 integrin mRNA in mammary cells. J. Cell Sci. **108**, 595–607
45 Schaller, M. D. and Parsons, J. T. (1995) pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. Mol. Cell Biol. **15**, 2635–2645
46 Johnson, L. N., Noble, M. E. and Owen, D. J. (1996) Active and inactive protein kinases: structural basis for regulation. Cell **85**, 149–158
47 Reference deleted
48 Toutant, M., Studler, J. M., Burgaya, F., Costa, A., Ezan, P., Gelman, M. and Girault, J. A. (2000) Autophosphorylation of Tyr397 and its phosphorylation by Src-family kinases are altered in focal-adhesion-kinase neuronal isoforms. Biochem. J. **348**, 119–128
49 Thomas, S. M., Soriano, P. and Imamoto, A. (1995) Specific and redundant roles of Src and Fyn in organizing the cytoskeleton. Nature (London) **376**, 267–271
50 Vuori, K., Hirai, H., Aizawa, S. and Ruoslahti, E. (1996) Introduction of $p130^{cas}$ signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. Mol. Cell Biol. **16**, 2606–2613
51 Schlaepfer, D. D., Jones, K. C. and Hunter, T. (1998) Multiple Grb2-mediated integrin-stimulated signaling pathways to ERK2/mitogen-activated protein kinase: summation of both c-Src- and focal adhesion kinase-initiated tyrosine phosphorylation events. Mol. Cell Biol. **18**, 2571–2585
52 Klemke, R. L., Leng, J., Molander, R., Brooks, P. C., Vuori, K. and Cheresh, D. A. (1998) CAS/Crk coupling serves as a 'molecular switch' for induction of cell migration. J. Cell Biol. **140**, 961–972
53 Webster, M. K. and Donoghue, D. J. (1996) Constitutive activation of fibroblast growth factor receptor 3 by the transmembrane domain point mutation found in achondroplasia. EMBO J. **15**, 520–527

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APPENDIX C

Article in Press.

"FAK regulates biological processes important fro the pathogenesis of cancer"

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FAK regulates biological processes important for the pathogenesis of cancer

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Key words: focal-adhesion kinase, signaling, cancer

Summary

Since its initial discovery as a substrate and binding partner for the Src oncogene, a role for the focal adhesion kinase (FAK) in cancer has been speculated. In this review the clinical evidence correlating FAK overexpression with cancer and the experimental evidence demonstrating that FAK can control some phenotypes associated with cancer will be discussed. In addition, the emerging theme of interactions between the FAK and growth factor signaling pathways will be described. The evidence presented in this review provides a compelling case for a role for FAK in the pathology of human cancer.

Introduction

The focal adhesion kinase (FAK) was initially identified as a tyrosine phosphorylated protein in cells transformed by the Src oncogene, and was later found to associate with Src via an SH2 domain mediated interaction [1–3]. Using a PCR based cloning strategy, FAK was independently identified as a tyrosine kinase that was expressed in a high grade sarcoma [4]. Thus, since its original isolation from both a model system for cancer and tumor tissue, FAK has been considered a candidate signaling protein that might function in the development of human cancer.

The FAK gene is located on human chromosome 8q24, in proximity to the c-myc locus [5,6]. FAK is an essential gene product as FAK null mice exhibit embryonic lethality [7]. Under normal conditions, FAK controls a number of biological processes including cell spreading, proliferation, survival and motility [8,9]. Deregulation of several of these processes is associated with malignancy. It is therefore easy to envision how aberrant regulation of FAK signaling could contribute to the progression of a cancerous phenotype.

In normal cells, tyrosine phosphorylation and activation of the catalytic activity of FAK are regulated by many stimuli, but the major regulators of FAK activity are the integrins, transmembrane receptors for proteins of the extracellular matrix [8,9]. Upon integrin-dependent cell adhesion, FAK co-localizes with the integrins at focal adhesions, sites of close contact with the extracellular matrix, and becomes tyrosine phosphorylated and activated. Changes in integrin expression have been observed in many cancers [10–12]. In a number of experimental systems, modulation of integrin expression has altered the phenotypes of cancer cells [13]. Integrin-mediated signaling events are very likely responsible for the phenotypic changes observed. As a major integrin regulated signaling molecule, FAK may function downstream of integrins in altering the phenotypes of cancerous cells, a hypothesis that remains to be tested.

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There are four major modular domains within FAK [8,9]. At the N-terminus is a FERM domain, which in many proteins mediates protein-protein interactions, often between a cytosolic protein and the cytoplasmic domain of a transmembrane protein. Centrally located within FAK is the catalytic domain. The C-terminal noncatalytic domain of FAK is comprised of two domains. The region proximal to the catalytic domain is rather nondescript, but contains two proline-rich sequences. At the C-terminus of the protein is the focal adhesion targeting (FAT) sequence, the region of FAK that mediates its discrete localization to focal adhesions. Interestingly, the entire C-terminal, non-catalytic domain of FAK (called FRNK) is autonomously expressed in some cell types, and has been utilized as a dominant negative mutant to elucidate FAK function. There are several sites of tyrosine phosphorylation on FAK, including the major site of autophosphorylation (Y397), and sites that serve as substrates for Src family kinases, which reside in the activation loop of FAK (Y576/577) and the FAT sequence (Y925).

FAK contains binding sites for many signaling proteins that play an important role in normal FAK function (Figure 1). The FERM domain of FAK has been reported to directly associate with the cytoplasmic tail of integrins *in vitro*, and either directly or indirectly with the cytoplasmic domains of the EGF receptor and PDGF receptor [14–16]. Further, the FERM domain of FAK mediates interactions with a cytoplasmic tyrosine kinase Etk/Bmx and with the cytoskeletal protein ezrin [17,18]. The FAT sequence contains a binding site for paxillin, a tyrosine phosphorylated adaptor protein that recruits multiple signaling molecules into complex, including the Crk adaptor protein, via an SH2 domain mediated interaction [19]. The C-terminal proline-rich regions serve as docking sites for the SH3 domains of several proteins, including Cas, another tyrosine phosphorylated adaptor protein that associates with Crk [20]. Tyrosine phosphorylation of FAK regulates association with SH2 domain containing signaling proteins. The major site of autophosphorylation of FAK (Y397) is a binding site for the SH2 domains of Src, PI 3′-kinase and phospholipase C_γ. A site of tyrosine phosphorylation in the FAT sequence (Y925) serves as a binding site for the SH2 domain of Grb2 and links FAK to the MAP kinase pathway. Many of these proteins that directly or indirectly associate with FAK have been implicated in cancer. Src, Crk and PI-3 kinase have been isolated as oncogenes. In addition, altered activity of the cellular Src proto-oncogene has been found in a number of human tumors, including colon and breast cancer [21,22]. Although in most of these scenarios, the role of FAK has yet to be explored, these findings fueled speculation about the role of FAK in the development of cancer, despite the fact that FAK was simply guilty by association.

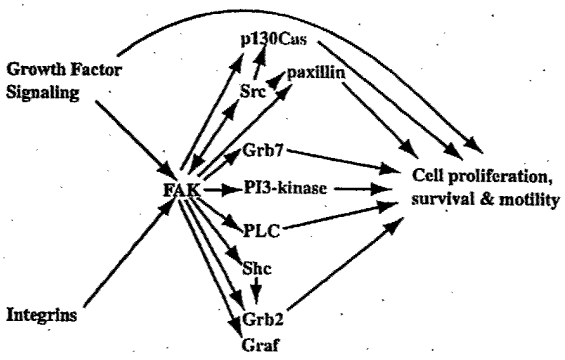

Figure 1. Schematic diagram of FAK controled signaling pathways. Stimulation of growth factor receptors and integrins in part converge upon FAK. Multiple binding partners/substrates of FAK may be involved in the control of biological processes that are aberrantly regulated in malignancies.

Several lines of investigation suggest that FAK and growth factor receptor signaling pathways are connected. As described above, the FERM domain of FAK mediates a direct or indirect interaction between FAK and several growth factor receptors [15,16]. In some systems, stimulation with growth factors can stimulate tyrosine phosphorylation of FAK [9]. Growth factors promote chemotactic motility and FAK is required for EGF and PDGF induced cell migration of mouse embryo fibroblasts [15]. Finally, it is now clearly established that efficient activation of biochemical signals following growth factor stimulation requires a second signal that is generated by integrin-dependent cell adhesion [23]. FAK was recently identified as a key signaling protein mediating the cross talk between adhesion-dependent signaling and growth factor receptors [24]. Given the important role of growth factor

receptors in the development of cancer, this is another potential link between FAK and cancer. The interplay between FAK and growth factor receptors in pathogenesis of cancer is beginning to be explored.

Correlative evidence: Overexpression of FAK in tumors

Although FAK itself has not been demonstrated to be an oncogene, elevated FAK protein levels have been reported in tumors of broad tissue origin (Table 1). FAK overexpression occurs in epithelial tumors of the breast [25–27], colon [25–29], thyroid [30], prostate [31,32], oral cavity [33], liver [25,27], stomach [27] and ovary [34]. In addition, FAK is upregulated in tumors of mesenchymal origin, including muscle [25] and glial cells [25,35–38]. Congruent with these observations in tumors, elevated FAK expression has been observed in tumor-derived cell lines in comparison to 'normal' cell lines (Table 2).

The overexpression of FAK in cancer may be the result of deregulation at several levels. The amount of FAK mRNA was higher in metastatic leiomyosarcomas relative to low grade leiomyosarcomas [4]. FAK mRNA levels were also elevated in colon, breast, prostate and hepatocellular carcinomas, and this was correlated with elevated protein expression [31,39,40]. FAK gene dosage is elevated in squamous cell carcinomas of

Table 1. FAK expression in tumors

Tissue	N	B	PI	I	M	Method	Reference
Breast		–		+++	++++	Northern	[39]
	+/–			+++	++++	Western	[25]
		+	+++	+++		IHC	[26]
	+/–			+/+++		IHC	[27]
Colon	–	+		++++	+++++	Northern	[39]
	+	–	+++	+++	+++	Western	[25]
	+				+++	Western	[28]
		+	+++	+++		IHC	[26]
	+			+++	+	Western, IHC	[29]
	+/–			++/+++		IHC	[27]
Thyroid	+	+		+/+++***	++++	Western	[30]
Prostate	+/–	+		+	+++	RT-PCR, Western	[31]
	+/+++	+/+++	+++	+++	+++	IHC	[32]
Oral cavity**	–/++		+/+++	+/++++		IHC	[33]
Liver	–			+++		Western	[25]
	+			+++++		RT-PCR	[40]
	+/–			++/+++		IHC	[27]
Stomach	+/–			++/+++		IHC	[27]
Ovary	+			++++		Western	[34]
Sarcoma**	++	+/–		++++		Western	[25]
Brain/astrocytes	–	–	+	–	+++++	Western	[35]
	–		–	+	++	IHC	[364]
		–	++	++	++	IHC	[7]
	+			+++		Western	[38]
Head neck				>2 copies		FISH	[6]

Expression or amplification of FAK in tumors and normal tissue is indicated. Expression levels are relative within the cited experiment and is defined using arbitrary units. N, normal; B, benign; PI, preinvasive; M, metastatic; IHC, immunohistochemistry; RT-PCR, reverse transcriptase polymerase chain reaction; FISH, fluorescent *in situ* hybridization.

** Evidence is compiled from various sarcomas or various oral cavity carcinomas.

*** Evidence from two types of invasive tumors: locally invasive/highly aggressive, metastatic.

Table 2. FAK expression in cancer cell lines

Cell/Cell line	Origin	Description	Fak level*	Evidence	Reference
Keratinocyes		Normal	2 copies	FISH	[6]
Various SCC lines	Head and neck	Invasive	4–6 copies	FISH	[6]
Various lung cancer lines			3–10 copies	FISH	[6]
Various colon cancer lines			3–10 copies	FISH	[6]
Various breast cancer lines			4–6 copies	FISH	[6]
T lymphocytes		Normal	Lower	Western	[83]
Various leukemia lines	T-cells	Transformed	Higher	Western	[83]
B lymphocytes		Normal	Lower	Western	[83]
Various lymphoma lines	B-cells	Transformed	Higher	Western	[83]
RCEC	Renal cortex epithelium	Normal	Lower	mRNA blot	[84]
Caki-2	Renal carcinoma	Non-invasive	Intermediate	mRNA blot	[84]
Caki-1	Renal carcinoma	Metastatic	Higher	mRNA blot	[84]
LNCaP	Prostate	Low tumorigenicity	Lower	RT-PCR, Western, IHC	[31, 32]
DU-145	Prostate	Highly tumorigenic	Elevated	RT-PCR, Western, IHC	[31, 32]
PC-3	Prostate	Highly tumorigenic	Greatly Elevated	RT-PCR, Western, IHC	[31, 32]
NHF	Mesenchymal	Untransformed	Lower	Western	[25]
WI-38	Mesenchymal	Untransformed	Lower	Western	[25]
LS180	Colon cancer	Transformed	Intermediate	Western	[25]
LS174T	Colon cancer	Transformed	Intermediate	Western	[25]
COLO205	Colon cancer	Transformed	Intermediate	Western	[25]
BT474	Breast cancer	Transformed	Higher	Western	[25]
BT-20	Breast cancer	Transformed	Higher	Western	[25]
RD	Rhabdomyosarcoma	Transformed	Higher	Western	[25]

FAK expression in various cancer cell lines and some of the properties of the cell lines are shown. SCC, squamous cell carcinoma; FISH, fluorescent in situ hybridization; IHC, immunohistochemistry.
* Relative level of FAK within each study.

the head and neck, as well as squamous cell, breast, colon, and lung carcinoma cell lines, suggesting that FAK gene amplification may be a common feature in tumors. Although increased gene copy number was associated with increased FAK protein expression, the two were not strictly correlated, suggesting that an additional level of regulation may exist [6]. While it is possible that FAK gene amplification is the mechanism by which mRNA and protein levels are elevated in tumors, at present, there have been few studies to address this hypothesis.

Initial correlative studies, incorporating data from tumors of diverse tissue and cellular origin, revealed stage specific overexpression of FAK. Through Northern and Western blot analyzes of tumor homogenates, FAK mRNA and protein levels were shown to be absent or low in normal tissue and benign neoplasms, and upregulated in invasive and metastatic tumors [25,30,39]. In an *in vitro* model of colon cancer progression, increased FAK gene dosage was correlated with increased protein expression during the transition from non-invasive adenoma to invasive carcinoma [6]. In prostate tissues, a PCR-based strategy was used to show that FAK mRNA levels were elevated in metastatic tumors relative to normal tissue. A corresponding increase in FAK protein was also noted in metastatic tumors [31]. These early studies highlighted a potential role for FAK in tumor progression to a malignant phenotype, and prompted more rigorous studies of tumor FAK expression at the cellular level.

Immunohistochemical analysis (IHC) has

allowed for discrimination and comparison of FAK expression between tumor and adjacent normal tissues within the same tissue section. Because IHC provides information about FAK expression on a cellular level, it is more sensitive to slight changes in FAK levels than blot analyses. In the majority of cases, the observation that FAK was overexpressed in invasive and metastatic tumors relative to normal adjacent tissues was confirmed [26,32,33,36,37], although some exceptions exist [29]. A unique feature of IHC is that precise tumor staging and FAK expression can be assessed simultaneously. Using this approach, it has become clear that FAK is not only overexpressed in malignant tumors, but also in premalignant lesions. For example, FAK was expressed at low levels in normal oral tissues, and was upregulated in *in situ* and primary oral cavity carcinomas. The percentage of cells overexpressing FAK as well as the intensity of FAK staining within individual cells was elevated above normal levels. Furthermore, FAK expression was restricted to the proliferative basal cell layers of the normal stratified squamous epithelium but extended upwards towards the lumen and downwards into the stroma in some invasive tumors. Finally, this study was the first to describe the great heterogeneity in FAK staining that occurs within a single tumor and between different tumors of the same stage. Interestingly, subpopulations of cells exhibiting stronger FAK staining were observed in early tumors [33]. FAK expression was also variable yet clearly upregulated in premalignant and malignant colon and breast tumors in comparison to normal tissues, as assessed by IHC [26]. Most recently, Rovin et al. [32] have used IHC to study FAK expression and subcellular localization in prostate cancer. In normal prostate epithelium, FAK was highly expressed in the basal cell layer and weakly expressed in the secretory layer. Benign hypertrophic lesions did not have elevated FAK expression. However, preinvasive lesions had strong FAK staining and cells expressing FAK were not restricted to the basal layer. Invasive lesions had strong staining which was heterogeneous within the tumor. In metastatic tumors, FAK staining was strong and homogenous throughout the tumor [32]. These studies clearly show that FAK upregulation is an early event in invasive tumor development. Furthermore, the fact that FAK expression is maintained at high levels throughout progression implies a permissive and perhaps active role for FAK in the pathogenesis of cancer.

Experimental evidence: Alteration of FAK signaling alters phenotypes associated with cancer cells

FAK is overexpressed in preinvasive lesions and is sustained in invasive and metastatic tumors. In addition, the deregulation of processes that are normally regulated by FAK, namely adhesion-dependent cell growth, survival and motility, are critical aspects of tumor progression. These observations underscore the possibility that overexpression of FAK confers a selective advantage to tumor cells, and therefore contributes to tumor progression. However, the mechanisms by which FAK may influence tumor cell behavior remain unclear. Studies of FAK signaling in cell and animal model systems have provided insight into FAK function in tumors. Overexpression of FAK or a constitutively activated form of FAK (CD2FAK) has been used to increase FAK signaling in cells to model the observed elevation of FAK expression in tumors [41]. In contrast, the use of antisense oligonucleotides or the expression of dominant negative mutants of FAK, including FRNK (also known as FAK CD), have been used to inhibit FAK expression and signaling respectively [42,43]. FAK null cells and animal models have also been valuable tools to determine FAK function in cancer. In the following sections, we describe the experimental evidence that supports a role for FAK in tumor cell function and progression (Table 4).

Spreading and adhesion

Cell adhesion is an important component of cancer cell behavior, including its ability to migrate, invade and metastasize. FAK is a regulator of focal adhesion turnover and cell spreading in normal cells [8,9]. A correlation exists between FAK expression and the rate of cancer cell adhesion and spreading in culture (Table 3). The introduction of FAK into melanoma cells that usually grow in suspension caused the cells to

Table 3. Correlation between cancer cell phenotypes and FAK signaling

Type of cell	Treatment	FAK status	Biological effect	Reference
Melanoma	Arachidonic acid metabolite	Increased phosphorylation	Increased sprending	[85]
Melanoma		Increased expression	Increased spreading	[101]
Highly metastatic Prostate carcinoma	Genistein intake	Transient phosphorylation Transient binding to b1 integrin	Increased spreading Increased adhesion	[86]
Colonic adenonoma to carcinoma model		Increased expression with progression	Increased adhesion with progression	[102]
Colon adenocarcinoma	Retinoic acid	Increased expression	Increased adhesion with progression	[87]
Highly metastatic ovarian carcinoma (HM) vs. low metastatic ovarian carcinoma (LM)	FN adhesion	HM: Decreased expression (soluble pool); decreased phosphorylation LM: increased phosphorylation	Decreased matrix adhesion Decreased cell-cell adhesion	[88]
Non-tumorigenic Breast epithelia (NT) vs. Cancer/Tumorigenic Breast epithelia (T)	Cell density: low / high	NT: High / low phosphorylation T: High / high		[89]
Melanoma	Echistatin treatment	Dephosphorylation	Loss of adhesion	[90]
Breast carcinoma	IGFBP-1 or RGD treatment	Dephosphorylation	Loss of adhesion Apoptosis	[91]
Prostate carcinoma	RGD peptide treatment	Cleaved	Apoptosis	[92]
Normal Breast epithelia	Bcl2 expression	Constitutive phosphorylation	Inhibition of anoikis	[93]
Hepatocellular carcinoma	Expression of a6b1 deletion mutant	Decrease phosphorylation Decreased association with Grb2	Decreased migration	[94]
Melanoma	FN stimulus	Increased expression	Increased migration	[95]
Highly tumorigenic Prostate carcinoma (HT) vs. Poorly tumorigenic Prostate carcinoma		HT: Increased expression Constitutive phosphorylation (Y861)	Increased migration	[52]
Epidermoid carcinoma (EGFR expressors)	EGF treatment	Decreased phosphorylation (early) Decreased activity	Loss of adhesion Increased motility Increased invasion	[71]
Colonic adenonoma to carcinoma model	EGF treatment	Increased expression Rapid phosphorylation	Increased invasion	[70]
Glioma	Temozolomide treatment	Cleaved	Decrease invasion (irradiation-induced)	[96]
Highly tumorigenic Prostate carcinoma (HT) vs. Poorly tumorigenic Prostate carcinoma		HT: Increased expression Increased phosphorylation	Increased invasion	[31]
Transformed Fibroblasts	v-Src expression	Cleaved (by calpain)	Decrease adhesion Increased motility Increased adhesion-independent growth	[47]
Transformed Fibroblasts	v-Src expression	Increased activity	Increased adhesion-independent growth	[97]
Normal Breast epithelia (N) Breast carcinoma (C) Metastatic Breast Carcinoma (M)	Suspension	N: Low phosphorylation / high in matrigel M: Increased phosphorylation and activity	Adhesion-independent growth	[68,69]
Metastatic Breast carcinoma (M) vs. normal epithelia	Suspension	M: Constitutive phosphorylation	Metastasis	[98]
Transformed Fibroblasts	v-Src expression	Increased phosphorylation	Induction of metastatis	[99]

In a number of cancer cells, specific stimuli produce changes in FAK signaling, including changes in the level of FAK expression, tyrosine phosphorylation and proteolytic cleavage. In many instances, these changes in FAK signaling have been correlated with changes in a biological response that may be controlled by FAK.

adhere to the extracellular matrix. In contrast, inhibition of FAK expression by antisense in FAK-expressing melanomas and breast cancer cells caused the cells to detach [44,45]. Similarly, the expression of FAK CD caused irreversible loss of cell adhesion of human breast cancer and melanoma cells [43,46]. Interestingly, FAK CD has no effect on normal mammary epithelial cells. FAK cleavage by calpain was necessary for v-Src induced morphological transformation to a less adherent, more rounded phenotype [47,48]. Therefore, FAK signaling may be required for proper regulation of tumor cell adhesion, which may influence the ability of tumor cells to migrate and invade.

Anoikis survival: Adhesion Independent growth

Upon cell adhesion, engagement of the integrins by their extracellular matrix ligands promotes survival. When normal cells lose this adhesion signal, they undergo anoikis (apoptosis due to lack of cell adhesion). FAK signaling is an important component of the integrin-mediated survival signal [49]. As cancer cells progress to a malignant phenotype, they acquire the ability to survive in

Table 4. Experimental evidence linking FAK to cancer phenotypes

Cells	FAK status	Biological effect	Reference
Melanoma	Inhibit expression with antisense	Loss of cell adhesion	[44]
Breast cancer	Inhibit expression with antisense Cleave by FAK-CD	Irreversible loss of adhesion Apoptosis	[43,45,46]
Melanoma	Inhibit signaling with FRNK	Loss of cell adhesion	[98]
Trigeminal neurinoma	Degrade with cell rounding	Irreversible loss adhesion	[103]
Fibroblasts	Degrade with myc transformation	Apoptosis	[100]
NSCL cancer	Inhibit expression with antisense	Decrease adhesion independent growth	[51]
Fibroblasts (Ras transformed)	Constitutively activate (CD2FAK)	Enhanced adhesion independent growth	[24]
MDCK	Increase expression by overexpression Inhibit signaling with FRNK	HGF-induced adhesion independent growth and invasion Inhibibiton of HGF-induced adhesion independent growth and invasion	[50]
Astrocytoma	Increase expression by overexpression	Increase migration Adhesion independent growth Tumor in nude mice	[36]
MDCK	Constitutively activate (CD2FAK)	Acquisition of adhesion independent growth Tumors in nude mice Inhibition of anoikis	[49]
Prostate carcinoma	Inhibit signaling with FRNK	Inhibit migration	[52]
Prostate carcinoma (avb3 transfected)	Inhibit signaling with FRNK	Inhibit migration	[13]
Glioblastoma	Inhibit signaling with FAT	Inhibit EGF-mediated motility	[37]
Adenocarcinoma	Inhibit expression with antisense No expression (FAK–/–) Inhibit signaling with FRNK	Inhibit EGF-mediated motility and invasion	[53] *
Melanoma (highly metastatic)	Inhibit signaling with FRNK	Decrease migration Decrease invasion	[56]
Fibroblasts	No expression (FAK –/–)	Inhibit CD151-mediated migration and invasion	[55]
Epidermoid (EGFR expressors)	Inhibit signaling with FRNK	Enhanced EGF-mediated motility and invasion	[71]
Ovarian cancer	Inhibit expression with antisense	Inhibit invasion	[59,104,105] *
Melanoma	Inhibit signaling with FRNK	Decrease lung metastasis	[61] **
Carcinoma	Inhibit signaling with FRNK	Inhibit tumor growth	[64] ***
Papilloma	Decrease expression (FAK +/– mice)	Reduce tumor formation	[62]

In order to explore the role of FAK in cancer, FAK expression and/or signaling has been either impaired or enhanced in model systems for cancer. Alteration in FAK signaling in these systems produced defined changes in biological properties associated with the cancer phenotype.

* FAK also plays a role in MMP secretion/activation.

** correlates with changes in proliferation.

*** involvement of urokinase receptor signaling.

the absence of adhesion. FAK potentially contributes to adhesion independent growth by promoting cell cycle progression, proliferation and/or cell survival.

The overexpression of FAK in human malignant astrocytoma cells increased their adhesion independent growth in culture, which was associated with increased tumor cell growth [36]. Overexpression of FAK induced a slight increase in adhesion independent growth of MDCK cells

[50]. Further elevation of the FAK signal by expression of CD2FAK dramatically increased the growth of MDCK cells in soft agar. This may be due to increased survival, since CD2FAK promotes survival of MDCK cells in the absence of adhesion by inhibiting anoikis [49]. The ability of FAK to induce adhesion independent growth may be cell type specific, since CD2FAK was unable to induce adhesion independent growth in NIH-3T3 cells. Interestingly, CD2FAK enhanced the adhesion independent growth of Ras-transformed NIH-3T3 cells [24]. Furthermore, in the presence of both FAK and HGF, astrocytoma cells were able to grow in the absence of adhesion [50]. Thus, in certain scenarios FAK overexpression has been linked to adhesion independent growth.

Several studies have also investigated the role of endogenous FAK in adhesion-independent growth. The attenuation of FAK expression by antisense resulted in a loss of adhesion-independent growth of non-small cell lung cancer cells [51]. In our laboratory, the inhibition of FAK signaling by FRNK reduced the ability of breast cancer epithelial cells to grow in an adhesion-independent manner [V.G.-N. and M.D.S., unpublished observations]. Endogenous FAK in breast cancer cells functions in promoting cell survival, since the expression of FAKCD in breast cancer cells induced apoptosis even in cells that are viable in suspension through a process involving a death receptor and caspase 8 [43,46]. These studies suggest that endogenous FAK is required for cancer cell viability and adhesion independent growth.

Overall, these observations demonstrate that FAK-mediated signaling events can contribute to the process through which a cancer cell acquires adhesion-independent growth. However, this effect may be cell type specific and/or require the cooperation of another oncogene, e.g. ras.

Motility and invasion to metastasis

Metastasis is multi-step process that results from the convergence of a variety of cellular processes. Some of these processes include detachment from the extracellular matrix, cell migration, invasion into the surrounding stroma and through vessel walls, and survival in an adhesion-independent manner in a foreign environment. Invasion and metastasis are in general associated with increased motility in culture. Furthermore, the ability of tumor cells to migrate, invade and metastasize is associated with increased FAK expression (Table 3). For example, prostate carcinoma cells (PC3), which are highly tumorigenic, are also highly migratory, whereas poorly tumorigenic prostate carcinoma cells (LNCaP), are relatively less motile. PC3 cells have a higher level of FAK protein, autophosphorylation and catalytic activity compared to LNCaP cells. Introduction of the FAK dominant negative FRNK into PC3 cells inhibited migration, suggesting that FAK is an important regulator of motility in these cells [52]. Increased migration of LNCaP cells has been achieved by expression of the β3 integrin subunit. Interestingly, the motility is dependent on FAK signaling, since the introduction of FRNK in the β3-expressing LNCaP cells inhibited migration [13]. Inhibiting FAK expression by antisense and FAK signaling by FRNK also reduced EGF induced motility of adenocarcinoma cells [53]. Thus, in a number of different cancer cell lines, FAK is required for motility.

Whereas FAK inhibition reduced tumor cell motility, overexpression of FAK can have a positive effect on cancer cell motility. The expression of FAK in human malignant astrocytic tumor cells increased their migration. This change was associated with increased phosphorylation of p130cas, a known mediator of FAK motility, and increased localization of exogenous FAK to focal adhesions [36]. FAK also functions in the control of cell motility by CD151, a member of the TM4SF family of proteins [54]. Overexpression of CD151 resulted in increased motility, invasion and metastasis of human colon, glioblastoma, and fibrosarcoma cancer cell lines [55]. Interestingly, expression of CD151 increased the migration of FAK+/+ fibroblasts but has no effect in FAK −/− cells, suggesting that FAK is an essential mediator of the CD151-induced motility response [55]. These observations, in conjunction with the correlation between cancer cell motility and the level of FAK expression, underscores the importance of FAK signaling in mediating the migratory ability of tumor cells.

Invasion is a complex process that requires cells to migrate through the basement membrane and into the underlying stroma. The invasive potential

of tumor cells correlates with their aggressiveness and their ability to metastasize. Inhibition of FAK expression using antisense or signaling with FRNK expression reduced EGF-induced invasion in adenocarcinoma cells [53]. The invasiveness of highly metastatic melanoma and glioblastoma cells can also be inhibited by FRNK and the FAT domain of FAK [56,57]. Furthermore, expression of CD151 increased the invasion of FAK+/+ fibroblasts but had no effect on FAK−/− cells [55]. FAK may regulate invasion by promoting cancer cell motility and may also regulate secretion of matrix metalloproteinases (MMPs), which degrade the basement membrane. MMP activity has been correlated with elevated cell migration and invasion [58]. Interestingly, FAK signaling has been found to mediate MMP9 secretion [53,59,60]. The use of antisense and the introduction of FRNK to decrease FAK expression and signaling in adenocarcinoma cells inhibited their migratory and invasive activity and was associated with a decrease in MMP9 secretion [53]. Thus, FAK signaling may act on MMP9 expression or secretion suggesting a mechanism through which FAK can influence the invasion of tumor cells.

Although multiple studies have examined the role of FAK in controlling motility and invasion, there have been few reports of the role of FAK in metastasis. Inhibition of FAK signaling by FRNK expression in melanoma cells inhibited metastatic colonization of the lung by these cells [61]. Although this observation implies that FAK is involved in metastasis, additional studies are needed. Regardless of the paucity of studies addressing the role of FAK in metastasis, it has been clearly established that FAK regulates cell motility and invasion in cultured tumor cells. In addition, in tumors, a correlation exists between FAK overexpression and invasion and metastasis. Therefore, it is very likely that FAK may play a critical role in tumor invasion and metastasis.

FAK and tumor growth

Despite the preponderance of evidence that FAK regulates normal and tumor cell proliferation, survival, motility and invasion in culture, it can not be presumed that FAK plays similar roles in actual tumors. In a handful of studies, the effects of FAK on tumor growth in mice have been assessed. The conclusions suggest that FAK may influence formation, growth, and metastasis of tumors.

In a well established model for skin carcinogenesis, FAK heterozygous mice showed reduced benign papilloma formation, but no difference in the rate of conversion to malignant carcinoma [62]. This implies that FAK dosage affects the ability of tumors to form. In a cell culture model system, the expression of CD2FAK in MDCK cells rescued the cells from anoikis and promoted growth in soft agar. When injected into nude mice, CD2FAK expressing MDCK cells were able to form tumors [49]. Similarly, astrocytoma cells overexpressing FAK formed larger tumors in nude mice than tumors derived from the parental cell line [36]. Elevated tumor size was associated with an increased rate of cell proliferation and no change in the rate of apoptosis. Further, expression of a hyperactive mutant of FAK, SuperFAK [63], in the T47D breast cancer cell line resulted in an increase in the size of tumors that form in nude mice [V.G.-N. and M.D.S., unpublished observations]. Conversely, FRNK expression inhibited the growth of human carcinoma cells into tumors in nude mice [64]. Again, the effect was on the rate of cell proliferation and not the rate of apoptosis. These studies suggest that in tumors, FAK may promote tumor growth through protection from anoikis and/or expedition of cell cycle progression. Finally, FRNK expression in melanoma cells reduced their ability to metastasize in a model system for evaluating metastatic colonization of the lung, supporting a role for FAK in the end stages of tumor progression [61]. Although only a few studies have been published, these results support the hypothesis that FAK not only controls a number of cancer cell phenotypes in cells in culture, but that FAK may play a pivotal role in the initiation and progression of tumor formation and metastasis.

Crosstalk between growth factor receptors and FAK

In normal and cancer cells, soluble factors acting through diverse receptors regulate the phosphorylation and activation of FAK [9,65–67]. Furthermore, emerging evidence suggests that growth factor signaling pathways, which are known to be

deregulated in cancer, crosstalk with FAK to send biochemical signals and influence cell behavior [23]. Therefore, although overexpression of wild type FAK is only weakly tumorigenic [50], FAK may contribute to tumor progression through cooperation with well-known oncogenes. For example, a constitutively activated FAK, CD2FAK, did not confer adhesion independent growth upon fibroblasts. However, CD2FAK enhanced the anchorage independent growth of Ras-transformed fibroblasts [24]. Herein we discuss the experimental evidence that crosstalk between FAK and the EGF and HGF signaling pathways is important for the induction of changes in tumor cell behavior.

ErbB2 overexpression and activation causes growth factor- and adhesion-independent growth, as well as invasion of normal human mammary epithelial cells [68,69]. Interestingly, FAK co-immunoprecipitated with ErbB2 in normal mammary epithelial cells [69]. Thus, the biological effects of ErbB2 overexpression could be due in part to crosstalk with FAK. In an *in vitro* model of colon cancer progression, the transition from adenoma to carcinoma was associated with FAK upregulation. EGF treatment induced FAK phosphorylation, c-Src activation, and invasion into a reconstituted basement membrane in the carcinoma cells, but not the adenoma cells [70]. Conversely, inhibition of FAK expression or FAK signaling inhibited EGF-induced motility of glioblastoma and adenocarcinoma cells [53,57], as well as invasion of adenocarcinoma cells [53]. Crosstalk between integrin and EGF signaling pathways during EGF-induced cell migration and invasion led to dynamic changes in FAK phosphorylation which were associated with changes in cell behavior [71]. Contrary to previous conclusions, FAK dephosphorylation, rather than phosphorylation, was required for motility and invasion in this system. Furthermore, inhibition of FAK signaling by expression of FRNK enhanced EGF-induced motility and invasion [71]. FAK may also cooperate with EGF to promote cell survival. The introduction of FAK CD into breast cancer epithelial cells causes irreversible loss of cell adhesion, FAK cleavage and cell death. However, in EGFR overexpressing breast cancer epithelial cells, although FAK CD still induces cell detachment, it is unable to cause FAK cleavage nor induce apoptosis [16]. Although the precise molecular details are not currently understood, the FAK and EGF signaling pathways clearly cooperate to effect tumor cell behavior in culture. The relevance of this crosstalk in tumors has yet to be explored.

Inappropriate activation of the hepatocyte growth factor/scatter factor/met (HGF/SF/Met) pathway has been linked to a variety of human tumors and their invasive and metastatic capabilities [72–76]. HGF activates FAK in normal and cancer cells [77–79], suggesting a possible synergism between FAK and HGF signaling. Hyperphosphorylation of FAK in response to HGF/SF correlated with increased motility of squamous cell carcinoma and small cell lung cancer cells [77,80], and increased growth and invasive capacity of mammary carcinoma cells [81]. Furthermore, NIH/3T3 cells that expressed oncogenic variants of the MET gene grew in suspension and formed tumors in nude mice. These phenotypes were associated with constitutive association of Src with Met, which led to increased FAK and paxillin phosphorylation [82]. Recently, the overexpression of FAK in combination with HGF treatment was shown to induce adhesion-independent growth and cell invasion. These effects were inhibited by FRNK expression, suggesting that FAK renders cells susceptible to transformation by HGF [50]. Although the data is mainly correlational at this point, these observations are consistent with a role for crosstalk between HGF/SF/Met and FAK in tumor cells. These studies show that FAK enhances tumor cell phenotypes through cooperation with growth factors in cell culture. Therefore, the cooperation of FAK and growth factors may be a mechanism for tumor progression.

FAK and tumor progression

How might FAK contribute to tumor progression? It is likely that depending on the type of cancer, FAK may be important at different points during tumor progression. As an example consider a model of tumor formation and progression in a generic columnar epithelium (Figure 2). In normal epithelium, FAK expression is low. Benign hyperplasias represent an accumulation of cells potentially due to changes in the regulation of the cell

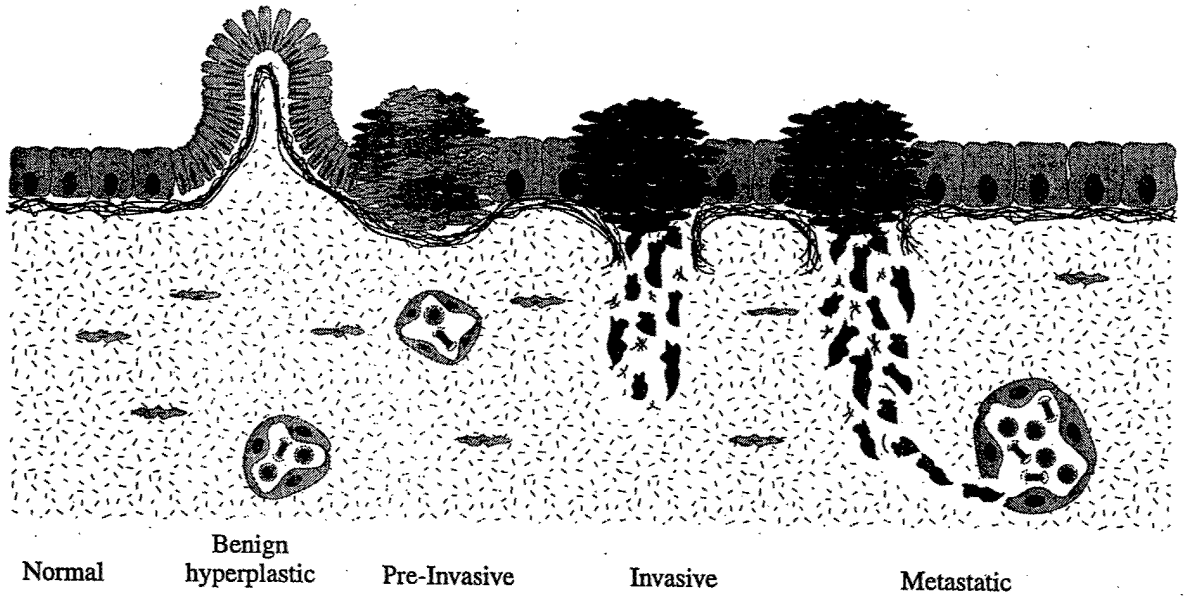

Figure 2. Model of FAK involvement in tumor progression. In normal epithelium and benign hyperplasias, FAK is expressed at low levels. In preinvasive, invasive and metastatic lesions, FAK is overexpressed (dark cells). Through its ability to regulate cell survival, growth, migration and invasion, FAK may influence tumor cell behavior and therefore promote malignancy.

cycle. Although FAK is not overexpressed in these lesions, the activity of endogenous FAK may be permissive for hyperplasia formation. Over time, cells within the hyperplastic lesion incur mutations that cause progression to a preinvasive phenotype. FAK overexpression is first observed at the preinvasive stage. FAK expression in preinvasive tumors is heterogeneous in some cases, suggesting that there is no selective pressure to express FAK. In other studies, FAK expression is relatively homogeneous throughout the tumor. At this point, cells no longer require adhesion to the basement membrane to survive and grow. Since FAK regulates anchorage independent growth and survival of cancer cells in culture and in some mouse model systems, FAK may confer these advantages in some tumors. In addition, as tumors progress, the rate at which they proliferate increases. Since overexpression of FAK causes an acceleration of tumor cell proliferation, FAK may be involved in driving proliferation in tumors.

Since in some preinvasive tumors, FAK is expressed in subpopulations of cells, it is tempting to suggest that these cells may be the invasive predecessors residing in preinvasive tumors. Progression to an invasive cancer requires degradation of the basement membrane and migration into the underlying stroma. FAK clearly regulates tumor cell motility and invasion in culture and may also regulate the secretion of matrix proteases. Therefore, at this stage, FAK may additionally contribute to the invasive capacity of the tumor. Finally, FAK is highly expressed in metastatic tumors and in prostate metastases FAK is homogenously expressed, suggesting that selection for FAK expressing cells may have occurred. In addition, there is one report experimentally linking FAK signaling to metastasis. Therefore FAK is also likely to function in later stages of cancer progression.

Conclusions

In the normal tissues that have been characterized, FAK expression is relatively low, whereas FAK is overexpressed in early preinvasive lesions and is highly expressed in metastatic lesions. Therefore, FAK expression could serve as a prognostic tool and may allow for accurate diagnosis and administration of appropriate treatments. In addition, FAK may be useful as a therapeutic target, since inhibition of FAK signaling can impair tumor growth and metastasis. In some studies, inhibition

of FAK expression/signaling induced apoptosis in cancer cells, but had little effect upon normal cells. This finding is particularly encouraging regarding the development of FAK as a therapeutic target.

A number of lines of investigation, both correlative and experimental, have implicated FAK as a potential player in the development and progression of cancer. Under normal conditions, FAK controls a number of important biological processes that become deregulated during the pathogenesis of cancer. Experimental evidence demonstrates that manipulation of FAK signaling in cancer cells alters the phenotype of these cells. Aberrant FAK expression in human cancers has also been extensively documented. The summation of these finding makes a very compelling case for a role for FAK in the pathogenesis of human cancer, and suggest that FAK might be a rational target for therapeutic intervention.

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References

1. Kanner SB, Reynolds AB, Vines RR, Parsons JT: Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. Proc Natl Acad Sci USA 87: 3328–3332, 1990
2. Schaller MD, Borgman CA, Cobb BS, Vines RR, Reynolds AB, Parsons JT: pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc Natl Acad Sci USA 89: 5192–5196, 1992
3. Cobb BS, Schaller MD, Leu TH, Parsons JT: Stable association of pp60src and pp59fyn with the focal adhesion-associated protein tyrosine kinase, pp125FAK. Mol Cell Biol 14: 147–155, 1994
4. Weiner TM, Liu ET, Craven RJ, Cance WG: Expression of growth factor receptors, the focal adhesion kinase, and other tyrosine kinases in human soft tissue tumors. Ann Surg Oncol 1: 18–27, 1994
5. Fiedorek FT Jr., Kay ES: Mapping of the focal adhesion kinase (Fadk) gene to mouse chromosome 15 and human chromosome 8. Mamm Genome 6: 123–126, 1995
6. Agochiya M, Brunton VG, Owens DW, Parkinson EK, Paraskeva C, Keith WN, Frame MC: Increased dosage and amplification of the focal adhesion kinase gene in human cancer cells. Oncogene 18: 5646–5653, 1999
7. Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T: Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature 377: 539–544, 1995
8. Schaller MD: Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim Biophys Acta 1540: 1–21, 2001
9. Schlaepfer DD, Hauck CR, Sieg DJ: Signaling through focal adhesion kinase. Prog Biophys Mol Biol 71: 435–478, 1999
10. Ruoslahti E: Fibronectin and its integrin receptors in cancer. Adv Cancer Res 76: 1–20, 1999
11. Mizejewski GJ: Role of integrins in cancer: Survey of expression patterns. Proc Soc Exp Biol Med 222: 124–138, 1999
12. Sanders RJ, Mainiero F, Giancotti FG: The role of integrins in tumorigenesis and metastasis. Cancer Invest 16: 329–344, 1998
13. Zheng DQ, Woodard AS, Fornaro M, Tallini G, Languino LR: Prostatic carcinoma cell migration via alpha(v)beta3 integrin is modulated by a focal adhesion kinase pathway. Cancer Res 59: 1655–1664, 1999
14. Schaller MD, Otey CA, Hildebrand JD, Parsons JT: Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. J Cell Biol 130: 1181–1187, 1995
15. Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, Schlaepfer DD: FAK integrates growth-factor and integrin signals to promote cell migration. Nat Cell Biol 2: 249–256, 2000
16. Golubovskaya V, Beviglia L, Xu LH, Earp III HS, Craven R, Cance W: Dual inhibition of focal adhesion kinase and epidermal growth factor receptor pathways cooperatively induces death receptor-mediated apoptosis in human breast cancer cells. J Biol Chem 277: 38978–38987, 2002
17. Chen R, Kim O, Li M, Xiong X, Guan JL, Kung HJ, Chen H, Shimizu Y, Qiu Y: Regulation of the PH-domain-containing tyrosine kinase Etk by focal adhesion kinase through the FERM domain. Nat Cell Biol 3: 439–444, 2001
18. Poullet P, Gautreau A, Kadare G, Girault JA, Louvard D, Arpin M: Ezrin interacts with focal adhesion kinase and induces its activation independently of cell-matrix adhesion. J Biol Chem 276: 37686–37691, 2001
19. Schaller MD: Paxillin: A focal adhesion-associated adaptor protein. Oncogene 20: 6459–6472, 2001
20. O'Neill GM, Fashena SJ, Golemis EA: Integrin signaling: A new Cas(t) of characters enters the stage. Trends Cell Biol 10: 111–119, 2000

21. Frame MC: Src in cancer: Deregulation and consequences for cell behavior. Biochim Biophys Acta 1602: 114–130, 2002
22. Biscardi JS, Tice DA, Parsons SJ: c-Src, receptor tyrosine kinases, and human cancer. Adv Cancer Res 76: 61–119, 1999
23. Schwartz MA, Ginsberg MH: Networks and crosstalk: Integrin signaling spreads. Nat Cell Biol 4: E65–E68, 2002
24. Renshaw MW, Price LS, Schwartz MA: Focal adhesion kinase mediates the integrin signaling requirement for growth factor activation of MAP kinase. J Cell Biol 147: 611–618, 1999
25. Owens LV, Xu L, Craven RJ, Dent GA, Weiner TM, Kornberg L, Liu ET, Cance WG: Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. Cancer Res 55: 2752–2755, 1995
26. Cance WG, Harris JE, Iacocca MV, Roche E, Yang X, Chang J, Simkins S, Xu L: Immunohistochemical analyses of focal adhesion kinase expression in benign and malignant human breast and colon tissues: Correlation with preinvasive and invasive phenotypes. Clin Cancer Res 6: 2417–2423, 2000
27. Su JM, Gui L, Zhou YP, Zha XL: Expression of focal adhesion kinase and alpha5 and beta1 integrins in carcinomas and its clinical significance. World J Gastroenterol 8: 613–618, 2002
28. Han NM, Fleming RY, Curley SA, Gallick GE: Overexpression of focal adhesion kinase (p125FAK) in human colorectal carcinoma liver metastases: Independence from c-src or c-yes activation. Ann Surg Oncol 4: 264–268, 1997
29. Ayaki M, Komatsu K, Mukai M, Murata K, Kameyama M, Ishiguro S, Miyoshi J, Tatsuta M, Nakamura H: Reduced expression of focal adhesion kinase in liver metastases compared with matched primary human colorectal adenocarcinomas. Clin Cancer Res 7: 3106–3112, 2001
30. Owens LV, Xu L, Dent GA, Yang X, Sturge GC, Craven RJ, Cance WG: Focal adhesion kinase as a marker of invasive potential in differentiated human thyroid cancer. Ann Surg Oncol 3: 100–105, 1996
31. Tremblay L, Hauck W, Aprikian AG, Begin LR, Chapdelaine A, Chevalier S: Focal adhesion kinase (pp125FAK) expression, activation and association with paxillin and p50CSK in human metastatic prostate carcinoma. Int J Cancer 68: 164–171, 1996
32. Rovin JD, Frierson HF Jr., Ledinh W, Parsons JT, Adams RB: Expression of focal adhesion kinase in normal and pathologic human prostate tissues. Prostate 53: 124–132, 2002
33. Kornberg LJ: Focal adhesion kinase expression in oral cancers. Head Neck 20: 634–639, 1998
34. Judson PL, He X, Cance WG, Van Le L: Overexpression of focal adhesion kinase, a protein tyrosine kinase, in ovarian carcinoma. Cancer 86: 1551–1556, 1999
35. Zagzag D, Friedlander DR, Margolis B, Grumet M, Semenza GL, Zhong H, Simons JW, Holash J, Wiegand SJ, Yancopoulos GD: Molecular events implicated in brain tumor angiogenesis and invasion. Pediatr Neurosurg 33: 49–55, 2000
36. Wang D, Grammer JR, Cobbs CS, Stewart JE Jr., Liu Z, Rhoden R, Hecker TP, Ding Q, Gladson CL: p125 focal adhesion kinase promotes malignant astrocytoma cell proliferation *in vivo*. J Cell Sci 113 Pt 23: 4221–4230, 2000
37. Jones G, Machado J Jr., Tolnay M, Merlo A: PTEN-independent induction of caspase-mediated cell death and reduced invasion by the focal adhesion targeting domain (FAT) in human astrocytic brain tumors which highly express focal adhesion kinase (FAK). Cancer Res 61: 5688–5691, 2001
38. Hecker TP, Grammer JR, Gillespie GY, Stewart J Jr., Gladson CL: Focal adhesion kinase enhances signaling through the Shc/extracellular signal-regulated kinase pathway in anaplastic astrocytoma tumor biopsy samples. Cancer Res 62: 2699–2707, 2002
39. Weiner TM, Liu ET, Craven RJ, Cance WG: Expression of focal adhesion kinase gene and invasive cancer. Lancet 342: 1024–1025, 1993
40. Miyasaka Y, Enomoto N, Nagayama K, Izumi N, Marumo F, Watanabe M, Sato C: Analysis of differentially expressed genes in human hepatocellular carcinoma using suppression subtractive hybridization. Br J Cancer 85: 228–234, 2001
41. Chan PY, Kanner SB, Whitney G, Aruffo A: A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125FAK. J Biol Chem 269: 20567–20574, 1994
42. Richardson A, Parsons T: A mechanism for regulation of the adhesion-associated proteintyrosine kinase pp125FAK. Nature 380: 538–540, 1996
43. Xu LH, Yang X, Craven RJ, Cance WG: The COOH-terminal domain of the focal adhesion kinase induces loss of adhesion and cell death in human tumor cells. Cell Growth Differ 9: 999–1005, 1998
44. Maung K, Easty DJ, Hill SP, Bennett DC: Requirement for focal adhesion kinase in tumor cell adhesion. Oncogene 18: 6824–6828, 1999
45. Xu LH, Owens LV, Sturge GC, Yang X, Liu ET, Craven RJ, Cance WG: Attenuation of the expression of the focal adhesion kinase induces apoptosis in tumor cells. Cell Growth Differ 7: 413–418, 1996
46. Xu LH, Yang X, Bradham CA, Brenner DA, Baldwin AS Jr., Craven RJ, Cance WG: The focal adhesion kinase suppresses transformation-associated, anchorage-independent apoptosis in human breast cancer cells. Involvement of death receptor-related signaling pathways. J Biol Chem 275: 30597–30604, 2000
47. Carragher NO, Fincham VJ, Riley D, Frame MC: Cleavage of focal adhesion kinase by different proteases during SRC-regulated transformation and apoptosis. Distinct roles for calpain and caspases. J Biol Chem 276: 4270–4275, 2001
48. Carragher NO, Westhoff MA, Riley D, Potter DA, Dutt P, Elce JS, Greer PA, Frame MC: v-Src-induced modulation of the calpain-calpastatin proteolytic system regulates transformation. Mol Cell Biol 22: 257–269, 2002
49. Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY: Control

of adhesion-dependent cell survival by focal adhesion kinase. J Cell Biol 134: 793–799, 1996
50. Chan PC, Liang CC, Yu KC, Chang MC, Ho WL, Chen BH, Chen HC: Synergistic effect of focal adhesion kinase overexpression and hepatocyte growth factor stimulation on cell transformation. J Biol Chem, 2002
51. Leyton J, Garcia-Marin LJ, Tapia JA, Jensen RT, Moody TW: Bombesin and gastrin releasing peptide increase tyrosine phosphorylation of focal adhesion kinase and paxillin in non-small cell lung cancer cells. Cancer Lett 162: 87–95, 2001
52. Slack JK, Adams RB, Rovin JD, Bissonette EA, Stoker CE, Parsons JT: Alterations in the focal adhesion kinase/Src signal transduction pathway correlate with increased migratory capacity of prostate carcinoma cells. Oncogene 20: 1152–1163, 2001
53. Hauck CR, Sieg DJ, Hsia DA, Loftus JC, Gaarde WA, Monia BP, Schlaepfer DD: Inhibition of focal adhesion kinase expression or activity disrupts epidermal growth factor-stimulated signaling promoting the migration of invasive human carcinoma cells. Cancer Res 61: 7079–7090, 2001
54. Hemler ME: Specific tetraspanin functions. J Cell Biol 155: 1103–1107, 2001
55. Kohno M, Hasegawa H, Miyake M, Yamamoto T, Fujita S: CD151 enhances cell motility and metastasis of cancer cells in the presence of focal adhesion kinase. Int J Cancer 97: 336–343, 2002
56. Li X, Regezi J, Ross FP, Blystone S, Ilic D, Leong SP, Ramos DM: Integrin alphavbeta3 mediates K1735 murine melanoma cell motility *in vivo* and *in vitro*. J Cell Sci 114: 2665–2672, 2001
57. Jones G, Machado J Jr., Merlo A: Loss of focal adhesion kinase (FAK) inhibits epidermal growth factor receptor-dependent migration and induces aggregation of nh(2)-terminal FAK in the nuclei of apoptotic glioblastoma cells. Cancer Res 61: 4978–4981, 2001
58. Legrand C, Gilles C, Zahm JM, Polette M, Buisson AC, Kaplan H, Birembaut P, Tournier JM: Airway epithelial cell migration dynamics. MMP-9 role in cell-extracellular matrix remodeling. J Cell Biol 146: 517–529, 1999
59. Shibata K, Kikkawa F, Nawa A, Thant AA, Naruse K, Mizutani S, Hamaguchi M: Both focal adhesion kinase and c-Ras are required for the enhanced matrix metalloproteinase 9 secretion by fibronectin in ovarian cancer cells. Cancer Res 58: 900–903, 1998
60. Sein TT, Thant AA, Hiraiwa Y, Amin AR, Sohara Y, Liu Y, Matsuda S, Yamamoto T, Hamaguchi M: A role for FAK in the concanavalin A-dependent secretion of matrix metalloproteinase-2 and -9. Oncogene 19: 5539–5542, 2000
61. Abdel-Ghany M, Cheng HC, Elble RC, Pauli BU: Focal adhesion kinase activated by beta 4 integrin ligation to mCLCA1 mediates early metastatic growth. J Biol Chem 277: 34391–34400, 2002
62. McLean GW, Brown K, Arbuckle MI, Wyke AW, Pikkarainen T, Ruoslahti E, Frame MC: Decreased focal adhesion kinase suppresses papilloma formation during experimental mouse skin carcinogenesis. Cancer Res 61: 8385–8389, 2001
63. Gabarra-Niecko V, Keely PJ, Schaller MD: Characterization of an activated mutant of focal adhesion kinase: 'SuperFAK'. Biochem J 365: 591–603, 2002
64. Aguirre Ghiso JA: Inhibition of FAK signaling activated by urokinase receptor induces dormancy in human carcinoma cells *in vivo*. Oncogene 21: 2513–2524, 2002
65. Yamamura S, Hakomori S, Wada A, Igarashi Y: Sphingosine-1-phosphate inhibits haptotactic motility by overproduction of focal adhesion sites in B16 melanoma cells through EDG-induced activation of Rho. Ann NY Acad Sci 905: 301–307, 2000
66. Nurcombe V, Smart CE, Chipperfield H, Cool SM, Boilly B, Hondermarck H: The proliferative and migratory activities of breast cancer cells can be differentially regulated by heparan sulfates. J Biol Chem 275: 30009–30018, 2000
67. Lin VC, Ng EH, Aw SE, Tan MG, Ng EH, Bay BH: Progesterone induces focal adhesion in breast cancer cells MDA-MB-231 transfected with progesterone receptor complementary DNA. Mol Endocrinol 14: 348–358, 2000
68. Ignatoski KM, Ethier SP: Constitutive activation of pp125fak in newly isolated human breast cancer cell lines. Breast Cancer Res Treat 54: 173–182, 1999
69. Ignatoski KM, Maehama T, Markwart SM, Dixon JE, Livant DL, Ethier SP: ERBB-2 overexpression confers PI 3' kinase-dependent invasion capacity on human mammary epithelial cells. Br J Cancer 82: 666–674, 2000
70. Brunton VG, Ozanne BW, Paraskeva C, Frame MC: A role for epidermal growth factor receptor, c-Src and focal adhesion kinase in an *in vitro* model for the progression of colon cancer. Oncogene 14: 283–293, 1997
71. Lu Z, Jiang G, Blume-Jensen P, Hunter T: Epidermal growth factor-induced tumor cell invasion and metastasis initiated by dephosphorylation and downregulation of focal adhesion kinase. Mol Cell Biol 21: 4016–4031, 2001
72. Beviglia L, Matsumoto K, Lin CS, Ziober BL, Kramer RH: Expression of the c-Met/HGF receptor in human breast carcinoma: Correlation with tumor progression. Int J Cancer 74: 301–309, 1997
73. Ferracini R, Di Renzo MF, Scotlandi K, Baldini N, Olivero M, Lollini P, Cremona O, Campanacci M, Comoglio PM: The Met/HGF receptor is over-expressed in human osteosarcomas and is activated by either a paracrine or an autocrine circuit. Oncogene 10: 739–749, 1995
74. Di Renzo MF, Poulsom R, Olivero M, Comoglio PM, Lemoine NR: Expression of the Met/hepatocyte growth factor receptor in human pancreatic cancer. Cancer Res 55: 1129–1138, 1995
75. Di Renzo MF, Olivero M, Giacomini A, Porte H, Chastre E, Mirossay L, Nordlinger B, Bretti S, Bottardi S, Giordano S: Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. Clin Cancer Res 1: 147–154, 1995
76. Giordano S, Bardelli A, Zhen Z, Menard S, Ponzetto C, Comoglio PM: A point mutation in the MET oncogene abrogates metastasis without affecting transformation. Proc Natl Acad Sci USA 94: 13868–13872, 1997
77. Matsumoto K, Matsumoto K, Nakamura T, Kramer RH:

Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase (p125FAK) and promotes migration and invasion by oral squamous cell carcinoma cells. J Biol Chem 269: 31807–31813, 1994
78. Chen HC, Chan PC, Tang MJ, Cheng CH, Chang TJ: Tyrosine phosphorylation of focal adhesion kinase stimulated by hepatocyte growth factor leads to mitogen-activated protein kinase activation. J Biol Chem 273: 25777–25782, 1998
79. Beviglia L, Kramer RH: HGF induces FAK activation and integrin-mediated adhesion in MTLn3 breast carcinoma cells. Int J Cancer 83: 640–649, 1999
80. Maulik G, Kijima T, Ma PC, Ghosh SK, Lin J, Shapiro GI, Schaefer E, Tibaldi E, Johnson BE, Salgia R: Modulation of the c-Met/hepatocyte growth factor pathway in small cell lung cancer. Clin Cancer Res 8: 620–627, 2002
81. Rahimi N, Tremblay E, McAdam L, Park M, Schwall R, Elliott B: Identification of a hepatocyte growth factor autocrine loop in a murine mammary carcinoma. Cell Growth Differ 7: 263–270, 1996
82. Nakaigawa N, Weirich G, Schmidt L, Zbar B: Tumorigenesis mediated by MET mutant M1268T is inhibited by dominant-negative Src. Oncogene 19: 2996–3002, 2000
83. Whitney GS, Chan PY, Blake J, Cosand WL, Neubauer MG, Aruffo A, Kanner SB: Human T and B lymphocytes express a structurally conserved focal adhesion kinase, pp125FAK. DNA Cell Biol 12: 823–830, 1993
84. Jenq W, Cooper DR, Ramirez G: Integrin expression on cell adhesion function and up-regulation of P125FAK and paxillin in metastatic renal carcinoma cells. Connect Tissue Res 34: 161–174, 1996
85. Tang DG, Tarrien M, Dobrzynski P, Honn KV: Melanoma cell spreading on fibronectin induced by 12(S)-HETE involves both protein kina. J Cell Physiol 165: 291–306, 1995
86. Bergan R, Kyle E, Nguyen P, Trepel J, Ingui C, Neckers L: Genistein-stimulated adherence of prostate cancer cells is associated with the binding of focal adhesion kinase to beta-1-integrin. Clin Exp Metastasis 14: 389–398, 1996
87. Nakagawa K, Sogo S, Hioki K, Tokunaga R, Taketani S: Acquisition of cell adhesion and induction of focal adhesion kinase of human colon cancer Colo 201 cells by retinoic acid-induced differentiation. Differentiation 62: 249–257, 1998
88. Abe Y, Tsutsui T, Mu J, Kosugi A, Yagita H, Sobue K, Niwa O, Fujiwara H, Hamaoka T: A defect in cell-to-cell adhesion via integrin-fibronectin interactions in a highly metastatic tumor cell line. Jpn J Cancer Res 88: 64–71, 1997
89. Zhang L, Bewick M, Lafrenie RM: Role of Raf-1 and FAK in cell density-dependent regulation of integrin-dependent activation of MAP kinase. Carcinogenesis 23: 1251–1258, 2002
90. Staiano N, Garbi C, Squillacioti C, Esposito S, Di Martino E, Belisario MA, Nitsch L, Di Natale P: Echistatin induces decrease of pp125FAK phosphorylation, disassembly of actin cytoskeleton and focal adhesions, and detachment of fibronectin-adherent melanoma cells. Eur J Cell Biol 73: 298–305, 1997
91. Perks CM, Newcomb PV, Norman MR, Holly JM: Effect of insulin-like growth factor binding protein-1 on integrin signaling and the induction of apoptosis in human breast cancer cells. J Mol Endocrinol 22: 141–150, 1999
92. Chatterjee S, Brite KH, Matsumura A: Induction of apoptosis of integrin-expressing human prostate cancer cells by cyclic Arg-Gly-Asp peptides. Clin Cancer Res 7: 3006–3011, 2001
93. Lin HM, Lee YJ, Li G, Pestell RG, Kim HR: Bcl-2 induces cyclin D1 promoter activity in human breast epithelial cells independent of cell anchorage. Cell Death Differ 8: 44–50, 2001
94. Carloni V, Mazzocca A, Pantaleo P, Cordella C, Laffi G, Gentilini P: The integrin, alpha6beta1, is necessary for the matrix-dependent activation of FAK and MAP kinase and the migration of human hepatocarcinoma cells. Hepatology 34: 42–49, 2001
95. Akasaka T, van Leeuwen RL, Yoshinaga IG, Mihm MC Jr., Byers HR: Focal adhesion kinase (p125FAK) expression correlates with motility of human melanoma cell lines. J Invest Dermatol 105: 104–108, 1995
96. Wick W, Wick A, Schulz JB, Dichgans J, Rodemann HP, Weller M: Prevention of irradiation-induced glioma cell invasion by temozolomide involves caspase 3 activity and cleavage of focal adhesion kinase. Cancer Res 62: 1915–1919, 2002
97. Verderame MF, Guan JL, Woods Ignatoski KM: Transformation and pp60v-src autophosphorylation correlate with SHC-GRB2 complex formation in rat and chicken cells expressing host-range and kinase-active, transformation-defective alleles of v-src. Mol Biol Cell 6: 953–966, 1995
98. Kahana O, Micksche M, Witz IP, Yron I: The focal adhesion kinase (P125FAK) is constitutively active in human malignant melanoma. Oncogene 21: 3969–3977, 2002
99. Rodina A, Schramm K, Musatkina E, Kreuser ED, Tavitian A, Tatosyan A: Phosphorylation of p125FAK and paxillin focal adhesion proteins in src-transformed cells with different metastatic capacity. FEBS Lett 455: 145–148, 1999
100. Crouch DH, Fincham VJ, Frame MC: Targeted proteolysis of the focal adhesion kinase pp125 FAK during c-MYC-induced apoptosis is suppressed by integrin signaling. Oncogene 12: 2689–2696, 1996
101. van der Gaag EJ, Leccia MT, Dekker SK, Jalbert NL, Amodeo DM, Byers HR: Role of zyxin in differential cell spreading and proliferation of melanoma cells and melanocytes. J Invest Dermatol 118: 246–254, 2002
102. Brunton VG, Fincham VJ, McLean GW, Winder SJ, Paraskeva C, Marshall JF, Frame MC: The protrusive phase and full development of integrin-dependent adhesions in colon epithelial cells require FAK- and ERK-mediated actin spike formation: Deregulation in cancer cells. Neoplasia 3: 215–226, 2001
103. Marushige Y, Marushige K: Alterations in focal adhesion and cytoskeletal proteins during apoptosis. Anticancer Res 18: 301–307, 1998

104. Shibata K, Kikkawa F, Nawa A, Tamakoshi K, Suganuma N, Tomoda Y: Increased matrix metalloproteinase-9 activity in human ovarian cancer cells cultured with conditioned medium from human peritoneal tissue. Clin Exp Metastasis 15: 612–619, 1997

105. Shibata K, Kikkawa F, Nawa A, Suganuma N, Hamaguchi M: Fibronectin secretion from human peritoneal tissue induces Mr 92,000 type IV collagenase expression and invasion in ovarian cancer cell lines. Cancer Res 57: 5416–5420, 1997

APPENDIX D

Manuscript submitted.

"Interaction of the FERM and Catalytic Domains of FAK Promotes FAK Signaling"

Interaction of the FERM and Catalytic Domains of FAK Promotes FAK Signaling

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A hypothetical intramolecular interaction between the FERM and catalytic domains of FAK has been proposed to inhibit enzyme activity. We have now identified a direct physical interaction between the FAK FERM and catalytic domains. Point mutations in the FERM domain that disrupt this interaction had little effect upon catalytic activity *in vitro,* however, the interaction was required for signaling *in vivo.* The mutant exhibits reduced tyrosine phosphorylation and dramatically reduced Src family kinase binding. Further the ability of the mutant to transduce biochemical signals and to promote cell migration were severely impaired. Thus, in contrast to the original hypothesis, the FERM/catalytic domain interaction is required to promote tyrosine phosphorylation, Src binding and transmission of FAK signals.

The focal adhesion kinase, FAK, plays a major role in transducing signals downstream of integrins (Schaller, 2001; Schlaepfer et al., 1999). Upon integrin-mediated adhesion, FAK becomes tyrosine phosphorylated and activated. Additional signaling molecules, e.g. Src and PI3-kinase, are recruited into complex with FAK leading to the transduction of biochemical signals that control important biological processes. Integrin signaling via FAK regulates cell migration, proliferation and survival. FAK-dependent regulation of one or more of these processes is essential since *fak*$^{-/-}$ mice exhibit embryonic lethality (Ilic et al., 1995). Conversely, enhanced FAK signaling may lead to aberrant cell proliferation, survival, or migration, which may have pathological consequences in humans. For example, aberrant FAK signaling may contribute to cancer development and progression to metastatic disease (Schaller, 2001).

FAK contains three major domains, an N-terminal domain, a central catalytic domain and a C-terminal domain (Schaller, 2001; Schlaepfer et al., 1999). The C-terminal domain can be further subdivided into the focal adhesion targeting (FAT) sequence, comprising the C-terminal 140 amino acids of the protein, and the region between the catalytic domain and the FAT sequence. Focal adhesion-associated FAT sequence binding partners have been identified, and insight into the molecular basis of FAT sequence function was recently obtained from crystal and NMR structure analyses (Arold et al., 2002; Hayashi et al., 2002; Liu et al., 2002). The sequence between the catalytic domain and the FAT sequence contains docking sites for SH3 domain containing proteins, and thus serves as a scaffold for the recruitment of signaling proteins. Several sites of tyrosine phosphorylation play important regulatory roles in

FAK. Within the catalytic domain, two tyrosine residues in the activation loop regulate catalytic activity. The major site of autophosphorylation, tyrosine 397, lies just N-terminal to the catalytic domain and serves as a binding site for Src family kinases. While details regarding the function of the catalytic and C-terminal domains have been elucidated, fewer studies have examined the function of the N-terminal domain of FAK.

The N-terminal domain of FAK exhibits homology with FERM domains, a structurally conserved domain found in many proteins (Girault et al., 1999a). FERM domains are present in structural proteins such as talin and the ezrin/radixin/moesin (ERM) family of proteins (Girault et al., 1999a; Chishti et al., 1998) as well as signaling proteins like the JAK family of tyrosine kinases and several tyrosine phosphatases (Girault et al., 1999a; Chishti et al., 1998). Genetic lesions within the FERM domains of several proteins have been implicated in human disease. For example, defects in merlin/schwannomin, a member of the ERM family of proteins, leads to the development of neurofibromatosis type 2 (Gusella et al., 1999). Mutations in JAK3 have been identified in patients with severe-combined immunodeficiency syndrome, and mutations within myosin VIIa are linked to deafness (Ward et al., 2000; Zhou et al., 2001; Hasson, 1999).

FERM domains mediate protein-protein interactions, and two different paradigms of interaction have been described. First, FERM domains can mediate intermolecular interactions, usually allowing docking with the cytoplasmic tails of transmembrane proteins. Secondly, FERM domains function in either intramolecular or homophilic intermolecular interactions. The best-characterized FERM domain-mediated interactions are those of the ERM family of proteins. The ERM proteins bind via their FERM

domains to the cytoplasmic domain of transmembrane proteins, e.g. CD44 (Louvet-Vallee, 2000). Other examples of FERM domain mediated intermolecular interactions include binding of the talin FERM domain to the β subunit of integrins, and the interaction of the FERM domains of JAKs with the γc and gp130 subunits of cytokine receptors (Calderwood et al., 2002; Zhou et al., 2001; Hilkens et al., 2001). The FERM domains of the ERM proteins also bind intramolecularly to a site within the C-terminus (Louvet-Vallee, 2000). This interaction obscures the CD44 binding site within the FERM domain and an actin-binding site in the C-terminal tail. Regulation of this intramolecular interaction modulates ERM protein mediated intermolecular interactions. The FERM domain of JAK3 interacts with the C-terminal catalytic domain and this interaction is essential for catalytic activity (Zhou et al., 2001).

Like other FERM domains, the FAK FERM domain also mediates protein-protein interactions. Several binding partners have been identified, including the cytoplasmic tails of the β1 integrin subunit and growth factor receptors (Schaller et al., 1995; Sieg et al., 2000; Golubovskaya et al., 2002). The FERM domain of FAK also mediates an interaction with the cytoplasmic tyrosine kinase Etk/Bmx, and interacts weakly with the FERM domain of ezrin (Chen et al., 2001; Poullet et al., 2001). Several of these FERM domain mediated interactions may function in the control of cell motility as disruption of the FAK/growth factor receptor interaction impairs chemotaxis and mutants of Etk defective for FAK binding fail to promote cell motility in CHO cells (Sieg et al., 2000; Chen et al., 2001).

Since the FERM domain of FAK binds the cytoplasmic tails of transmembrane proteins, e.g. the EGF receptor (Sieg et al., 2000), we considered the possibility that, like

other FERM domains, the FERM domain of FAK might also mediate intramolecular interactions. We demonstrate here that the N-terminal FAK FERM domain can directly associate with full length FAK *in vitro*. The catalytic domain of FAK is required for its interaction with the FERM domain. A highly conserved, basic patch on the surface of the FAK FERM domain was identified by molecular modeling. Mutation of residues in this basic patch disrupted the ability of the FERM domain to associate with FAK *in vitro*. To assess the function of this interaction, substitutions were engineered in full length FAK. The mutant exhibited near wild type catalytic activity *in vitro*, but was defective for transducing biochemical and biological signals *in vivo*. These observations suggest that the FAK FERM domain regulates FAK signaling via a novel mechanism.

RESULTS

The FAK FERM domain binds FAK *in vitro*. Since FERM domains of other proteins mediate intramolecular interactions, the possibility that the FERM domain of FAK exhibited FAK-binding activity was explored. A GST fusion protein containing the N-terminal domain of FAK (including the entire FERM domain), immobilized to glutathione agarose beads, was incubated with lysates of CE cells overexpressing wild type, epitope tagged FAK. Bound proteins were analyzed by Western blotting using the KT3 monoclonal antibody, which recognizes the tag. FAK bound to the GST-FAK N-terminal domain fusion protein, but not to GST alone (Fig 1A). Since CAKβ exhibits extensive sequence similarity to FAK, the N-terminal domain of CAKβ was also examined for its ability to bind FAK. The CAKβ N-terminal domain also associated with FAK *in vitro*, although less FAK bound to the N-terminus of CAKβ than the N-terminus of FAK (Fig 1A). The ability of the N-termini of FAK and CAKβ to bind to CAKβ was also examined by incubating GST-FAK N-terminus and GST-CAKβ N-terminus fusion proteins, immobilized on glutathione agarose beads, with lysate from CE cells expressing exogenous, epitope-tagged CAKβ. Although CAKβ was efficiently expressed in CE cells, neither the N-terminal domain of FAK nor CAKβ bound to CAKβ (Fig 1A). Thus the N-terminus of FAK, and to a lesser extent the N-terminus of CAKβ, associate with exogenously expressed FAK *in vitro*, but not with CAKβ.

To further corroborate these findings, the ability of GST fusion proteins containing the N-terminus of FAK or CAKβ to associate with endogenous FAK and CAKβ was examined. Fusion proteins immobilized on glutathione agarose beads were incubated with lysates of CE cells or Rat-1 cells and bound FAK was detected by

Western blotting. The N-terminal domain of FAK associated with endogenous FAK *in vitro* (Fig 1B). The N-terminal domain of CAKβ bound very weakly to endogenous FAK and was not detectable in the exposure shown. No binding to GST alone was detected. Further, equal amounts of each GST fusion protein were used in the analysis (Fig. 1D). CAKβ is not expressed endogenously in CE cells, thus the interaction between the N-terminal domains of FAK and CAKβ with endogenous CAKβ was examined using lysates of Rat-1 cells. Similar to the results with exogenously expressed CAKβ, endogenous CAKβ failed to associate with either the N-terminal domain of FAK or CAKβ *in vitro* (Fig. 1C). These findings demonstrate that the N-terminus of FAK, and to a lesser extent the N-terminus of CAKβ, is capable of associating directly or indirectly with FAK *in vitro*, but that these N-terminal domains are not capable of associating with CAKβ.

The catalytic domain is required for N-terminal domain binding. The observation that the N-termini of FAK and CAKβ can associate with FAK, but not CAKβ, allowed the use of chimeric FAK/CAKβ molecules to map the region of FAK that interacts with the N-terminal domain. GST-FAK N-terminus and GST-CAKβ N-terminus fusion proteins immobilized on glutathione agarose beads were incubated with lysates of CE cells expressing various FAK/CAKβ chimeric proteins. The association of the chimeras with the N-terminus of FAK or CAKβ was determined by Western blotting using KT3. The catalytic domain of FAK was the principal determinant of the ability to bind the N-terminal domains of both FAK and CAKβ. Whereas chimera containing the FAK catalytic domain, FFF and CFC, associated with the N-termini of FAK and CAKβ *in*

vitro, chimera containing the catalytic domain of CAKβ, CCC and FCF, bound weakly if at all to the N-terminal domains of FAK and CAKβ (Fig. 2A). Each chimera failed to bind to GST alone.

A number of FAK mutants were expressed in CE cells and characterized for their ability to associate with the N-terminal domain of FAK *in vitro*. The major autophosphorylation site of FAK, tyrosine 397, is dispensable for the interaction with the N-terminal domain of FAK, since the 397F FAK mutant bound to the N-terminal domain (Fig. 2B). Catalytic activity of FAK was also not required for association with the FAK N-terminal domain *in vitro*, since two catalytically defective mutants of FAK, K454M and D564A, associated with the N-terminal domain (Fig. 2B and data not shown). Thus neither catalytic activity nor phosphorylation of FAK at its major autophosphorylation site was required for the interaction with FAK N-terminus *in vitro*.

Homology Modeling of the FERM Domain of FAK. Based upon sequence analysis, a FERM domain was identified within the N-terminal domain of FAK (Girault et al., 1999a). To gain insight into the function of the N-terminal domain, a molecular model of the FERM domain was created using the InsightII homology modeling program. The structures of several FERM domains were used as templates for homology modeling, and the best model was produced using the crystal structure of moesin as a template (Edwards and Keep, 2001). Residues flanking the FERM domain could not be modeled since the secondary structure of these regions was predicted to be neither α-helices nor β-strands.

FERM domains contain three lobes, F1, F2 and F3 (also referred to as the A, B and C subdomains) (Pearson et al., 2000; Hamada et al., 2000). The F1 subdomain resembles ubiquitin in its structure, the F2 subdomain has a protein fold similar to acyl-

CoA binding protein and the F3 subdomain is similar in structure to PH/PTB/EVH1 domains (Pearson et al., 2000; Hamada et al., 2000; Edwards and Keep, 2001). The first two strands of the ubiquitin-like domain of the FAK FERM domain could not be accurately modeled and were therefore omitted from the final structure (Fig. 3A).

Identification of N-Terminal Domain Residues Critical for FAK Binding. One of the striking features of the FAK FERM model was a large patch of basic residues on the surface at the tip of the F2 subdomain (Fig. 3B, top panel). These residues are highly conserved in FAK (Fig. 3B, middle panel) and are also conserved between FAK and CAKβ (Fig. 3B, bottom panel). Therefore this region was considered a candidate for the catalytic domain binding site within the FERM domain of FAK. To explore the role of these residues in FAK binding, alanine substitutions were made for several basic residues. Two mutants, one with alanine substitutions for K^{190} and K^{191} (KK) and one with alanine substitutions for K^{216}, K^{218} and R^{221} (KAKTLR), were created. These mutants were engineered into the GST-FAK N-terminal domain fusion protein construct, expressed, and tested for their ability to associate with FAK *in vitro*. Wild type FAK and several other FERM domain mutants efficiently associated with FAK *in vitro* (Fig. 4 and data not shown). In contrast, KK exhibited reduced binding relative to the wild type FERM domain and KAKTLR was devoid of binding activity (Fig. 4A). Comparable amounts of fusion proteins were used in these experiments (Fig. 4B). These findings suggest that conserved basic residues on the surface of the F2 subdomain of the FAK FERM domain are important for the *in vitro* interaction with the catalytic domain of FAK.

The FAK FERM domain binds directly to FAK. To determine if the interaction between the FAK FERM and catalytic domains was direct, purified, recombinant proteins were used. GST fusion proteins immobilized on beads were incubated with His-tagged FAK that was expressed and purified from insect cells. Binding of FAK to the GST fusion proteins was examined by Western blotting. Recombinant FAK bound to GST-N-FAK and very weakly bound to GST (Fig. 4C). Further, recombinant FAK bound very poorly to GST-KAKTLR, which contains point mutations that disrupt the interaction of the FERM domain with FAK *in vitro*. Equal amounts of the GST fusion proteins were used in this analysis (Fig. 4D). These findings demonstrate that the interaction of the FAK FERM domain with FAK *in vitro* was direct.

The FERM/catalytic domain interaction modulates FAK phosphorylation. In order to assess the consequences of disrupting the interaction between the N-terminal and catalytic domains of FAK, the KAKTLR mutant was selected for further characterization. The mutant was subcloned into the RCAS A retroviral vector and expressed in CE cells. KAKTLR could be expressed in CE cells to similar levels as wild type FAK, and correctly localized to focal adhesions (Fig. 5 and data not shown). Overexpression of FAK had little effect on the levels of cellular tyrosine phosphorylation, except that the exogenously expressed FAK protein now appears as the major tyrosine phosphorylated protein in the cell (Fig. 5A, top panel)(Schaller and Sasaki, 1997; Schaller and Parsons, 1995). Similarly, overexpression of KAKTLR had no effect on the phosphotyrosine levels of cellular proteins, however, tyrosine phosphorylation of KAKTLR was apparently reduced relative to wild type FAK (Fig. 5A). To verify this observation, exogenously expressed wild type FAK and KAKTLR were immunoprecipitated and

Western blotted with a phosphotyrosine antibody. KAKTLR exhibited reduced levels of phosphotyrosine relative to wild type FAK (Fig. 5B). The blot was stripped and reprobed to verify that equal levels of exogenously expressed FAK proteins were recovered. The major site of autophosphorylation of FAK, Y397, is an important regulatory residue. To determine if KAKTLR was defective for phosphorylation at this site, cell lysates were analyzed by Western blotting with a phosphospecific antibody recognizing phosphorylated Y397 (PY397). Phosphorylation of KAKTLR at Y397 was reduced relative to wild type FAK (Fig. 5C, top). A FAK Western blot verified that equal amounts of wild type FAK and KAKTLR were present in the lysates (Fig. 5C, bottom). These results demonstrate that the mutation disrupting the FERM domain/catalytic domain interaction leads to reduced tyrosine phosphorylation of FAK, particularly on tyrosine 397.

To further characterize KAKTLR, tyrosine phosphorylation in response to cell adhesion to fibronectin was examined. CE cells expressing wild type FAK or the KAKTLR mutant were trypsinized, held in suspension for 45 minutes, then plated on fibronectin for the indicated times. FAK and KAKTLR were immunoprecipitated and analyzed by Western blotting for phosphotyrosine. The phosphotyrosine content of both wild type FAK and KAKTLR was reduced when cells were held in suspension and increased upon cell adhesion to fibronectin (Fig. 6A). However, the level of tyrosine phosphorylation of KAKTLR was reduced relative to that of wild type FAK. Tyrosine phosphorylation was further analyzed using the PY397 phosphospecfic antibody. When held in suspension, phosphorylation of tyrosine 397 in FAK and KAKTLR was dramatically reduced (Fig. 6B). Upon adhesion to fibronectin, both become

phosphorylated at tyrosine 397. However, the level of phosphorylation at tyrosine 397 in KAKTLR was reduced relative to that of wild type FAK following adhesion to fibronectin. Differences in phosphotyrosine levels were not due to differences in expression or recovery of wild type and mutant FAK by immunoprecipitation (Fig. 6A & B). These results demonstrate that the KAKTLR mutation, which ablates the ability of the FERM domain to interact with FAK *in vitro*, also impairs tyrosine phosphorylation of FAK *in vivo* in response to a physiological stimulus.

The FERM/catalytic domain interaction is dispensable for catalytic activity. Since KAKTLR exhibits reduced tyrosine phosphorylation *in vivo,* and given the precedent that the FERM domain/catalytic domain interaction is critical for catalytic activity of JAK3 (Zhou et al., 2001), it seemed likely that the KAKTLR mutant would exhibit a catalytic defect. This possibility was examined by incubating FAK immune complexes in an *in vitro* kinase assay using poly[Glu,Tyr] as an exogenous substrate. Under these conditions, wild type FAK and the KAKTLR mutant catalyzed phosphorylation of the exogenous substrate. There was a slight reduction in the catalytic activity of the KAKTLR mutant relative to wild type, as measured in this assay (Fig. 7). Quantification by phosphorimager analysis revealed approximately a 20% reduction in the catalytic activity of the mutant. Western blotting the immune complexes revealed that equal amounts of wild type FAK and KAKTLR were immunoprecipitated (Fig. 7). Therefore, the mutation disrupting the FERM domain/catalytic domain interaction had very little effect upon kinase activity *in vitro*.

Perturbation of the FERM domain/catalytic domain interaction disrupts Src kinase binding. The KAKTLR mutant exhibits a defect in phosphorylation of tyrosine 397 *in*

vivo, a phosphorylation site that regulates binding to several SH2 domain-containing proteins, including Src family kinases. To explore the effect of the KAKTLR mutation upon association with Src kinases, wild type or mutant FAK proteins were expressed in CE cells and association with Fyn examined. Endogenous Fyn was immunoprecipitated from cell lysates and co-immunoprecipitated FAK was examined by Western blotting. Wild type FAK was readily co-immunoprecipitated with Fyn (Fig. 7C). In contrast, KAKTLR exhibited very weak binding to Fyn. Equal amounts of Fyn were immunoprecipitated from each of the lysates (Fig. 7C). These results demonstrate that the FAK FERM/catalytic domain interaction is required for the efficient phosphorylation of FAK at tyrosine 397 and the subsequent binding of Src family kinases.

Disrupting the FERM/catalytic domain interaction impairs signaling *in vivo*. Since KAKTLR exhibited reduced tyrosine phosphorylation *in vivo*, and tyrosine phosphorylation plays an important regulatory role in FAK signaling, the ability of the mutant to transmit biochemical signals *in vivo* was explored. Whereas overexpression of FAK in CE cells has little effect upon tyrosine phosphorylation of focal adhesion-associated proteins, inhibition of cellular phosphatases by treatment with vanadate induces a dramatic increase in tyrosine phosphorylation of focal adhesion-associated proteins in FAK overexpressing cells (Schaller and Sasaki, 1997; Schaller and Parsons, 1995). To explore transmission of downstream signals in this system, CE cells overexpressing wild type FAK or KAKTLR were treated with vanadate and cellular phosphotyrosine levels examined by Western blotting. Vanadate had little effect upon phosphotyrosine levels in mock transfected cells, but induced a dramatic increase in tyrosine phosphorylation in FAK overexpressing cells (Fig. 8A). In contrast, vanadate

treatment of KAKTLR overexpressing cells had little effect upon cellular phosphotyrosine levels, similar to the effect observed in mock transfected cells. These results demonstrate that disruption of the FERM domain interaction impairs the ability of FAK to transmit biochemical signals *in vivo*.

To examine the importance of the FAK FERM domain interaction with the catalytic domain in mediating biological functions controlled by FAK, the ability of KAKTLR to promote cell motility was examined. T47D/tva cells, a derivative of the T47D breast cancer cell line that is susceptible to infection with avian retroviral vectors, were used for this analysis (Gabarra-Niecko et al., 2002). T47D/tva cells were infected with the empty RCAS A vector or with RCAS A containing the wild type FAK or KAKTLR cDNAs. Expression levels of FAK and KAKTLR were comparable, as determined by Western blotting (data not shown). To measure motility, cells were suspended in serum-free medium and placed in the upper chamber of a transwell. Cell migration in response to a haptotactic stimulus, i.e. collagen coated on the underside of the transwell membrane, was examined (Figure 8B). Overexpression of FAK resulted in a 1.8 fold increase in the number of migrating cells compared to control cells. Overexpression of KAKTLR also increased the motility of T47D/tva cells. However, the mutant was defective for promoting cell motility, increasing migration by only 1.2 fold relative to the motility of control cells. This result demonstrates that the interaction between the FERM domain and the catalytic domain of FAK is not only required for biochemical signaling, but is also required for the control of biological responses by FAK.

DISCUSSION

The N-terminal region of FAK contains a FERM domain, a module that mediates protein-protein interactions. Like other FERM domains, the FAK FERM domain mediates interactions with several proteins including transmembrane proteins, e.g. the platelet-derived growth factor receptor, and cytosolic proteins, e.g. Etk (Sieg et al., 2000; Chen et al., 2001). We now report that the FAK FERM domain is also capable of mediating an "intramolecular" interaction, as has been reported for the FERM domains of other proteins, e.g. the ERM family of proteins and JAK3 (Louvet-Vallee, 2000; Zhou et al., 2001). This interaction appears to occur between a basic patch on the tip of the F2 subdomain of the FERM domain and an undefined site within the catalytic domain. The FERM domain/catalytic domain interaction is important for FAK signaling as disruption of the interaction impairs phosphorylation of FAK at tyrosine 397, recruitment of Src family kinases, tyrosine phosphorylation of downstream substrates and stimulation of cell migration. However, this interaction is dispensable for catalytic activity *in vitro*. Therefore the FAK FERM domain interaction with the catalytic domain of FAK represents a novel mechanism of regulating signal transduction.

To gain insight into the function of the FERM domain of FAK, a molecular model was generated. The molecular model has been useful in identifying highly conserved residues on the surface of the FAK FERM domain that may participate in protein-protein interactions. A basic patch at the tip of the acyl CoA binding protein-like subdomain (F2) of the FAK FERM domain mediates an interaction with FAK, as demonstrated by the KAKTLR mutant. Interestingly, two diverse lines of investigation suggest that a similar region of the merlin FERM domain may also mediate protein-protein interactions.

Point mutations in merlin lead to the development of neurofibromatosis type 2 (NF2). One mutation associated with NF2 is a cysteine for glycine substitution at position 197 of merlin. This mutation is unlikely to perturb structure, but is hypothesized to affect function by disrupting a protein interaction site (Shimizu et al., 2002). This point mutation occurs in a loop following the α-helix that contains the KAKTLR mutation in FAK. Analysis of Drosophila merlin has revealed a highly conserved blue box sequence that is important for merlin function in Drosophila. Deletion of the blue box or polyalanine substitution for the blue box sequences abolishes the function of Drosophila merlin (LaJeunesse et al., 1998). It is hypothesized that the blue box sequence mediates a protein-protein interaction important for merlin function. The blue box sequence is located in the loop immediately preceding the α-helix containing the KAKTLR mutation in FAK. These observations suggest that the tip of the F2 subdomain mediates protein-protein interactions in multiple FERM domains, although the molecular bases for these interactions are not conserved.

An intramolecular interaction between the FERM domain and catalytic domain of JAK3 has been reported and this interaction is required for catalytic activity (Zhou et al., 2001). In contrast, the FAK FERM/catalytic domain interaction is dispensable for catalytic activity, as measured in an *in vitro* kinase assay. However, the interaction between the FERM and catalytic domains of FAK is required for efficient phosphorylation of tyrosine 397, which lies within a loop of undefined structure between these two domains, and binding of Src family kinases. It is likely that the FERM domain mediated interaction alters the conformation of this loop, promoting phosphorylation of tyrosine 397 and Src kinase binding. According to this hypothesis, this interaction is

perturbed in the KAKTLR mutant and the phosphorylation/Src binding loop cannot be correctly oriented, resulting in reduced phosphorylation and Src kinase binding *in vivo.* Reduced binding to Src family kinases can greatly impair biochemical signaling *in vivo* and it is therefore likely that reduced signaling by the KAKTLR mutant is due to its reduced binding capacity for Src kinases (Schaller and Parsons, 1995; Frisch et al., 1996; Schlaepfer and Hunter, 1997). Since Src kinase binding to FAK is important for most biological functions, e.g. motility and cell survival (Frisch et al., 1996; Cary et al., 1996), this finding is also likely to be physiologically relevant.

How the interaction between the FAK FERM domain and catalytic domain is regulated is unknown, however, it may provide an addition level of regulation upon FAK signaling. For example, phosphorylation of tyrosine 397 is persistent in adherent cells, yet Src binding peaks transiently following cell adhesion (Schlaepfer et al., 1997). In addition, growth factor stimulation of cells can promote the association of Src with FAK without significantly altering the levels of phosphorylation of tyrosine 397 (Sieg et al., 2000). These observations could be explained by regulation of the FAK FERM domain/catalytic domain interaction, which is required for the association of Src kinases with FAK. Alternatively, this interaction may not be regulated, but rather serve a constitutive structural function to allow phosphorylation of tyrosine 397 and Src binding. Further studies are required to distinguish between these possibilities.

Although we have referred to the interaction of the FAK FERM and catalytic domains as "intramolecular", at present our results cannot distinguish between two models of interaction. The FERM domain interaction might be intramolecular (Fig 9A), or alternatively, the interaction could be intermolecular (Fig 9B). Our *in vitro* binding

experiments clearly demonstrate that the FAK FERM domain can bind to FAK via an intermolecular interaction. However, there is no evidence that FAK dimerizes *in vivo* (Toutant et al., 2002). There may be multiple interactions that promote FAK clustering *in vivo*, e.g. via C-terminal domain interactions with paxillin. FAK clustering could facilitate intermolecular interaction of the FAK FERM and catalytic domains. If the intermolecular interaction was relatively weak or transient in nature, it may not be detected under typical lysis conditions, consistent with the absence of evidence for FAK dimerization *in vivo*. Alternatively, the FAK FERM and catalytic domains could interact intramolecularly. Definitive evaluation of the intramolecular interaction model may require solution of the structure of FAK.

FERM domains mediate both intra- and intermolecular interactions, and in several well-documented cases, these interactions are not independent. Intramolecular interactions via FERM domains can block binding sites for other proteins and thus negatively regulate intermolecular interactions, as is the case for the ERM proteins (Louvet-Vallee, 2000). The intramolecular interaction between the FERM domain and catalytic domain of JAK3, and the intermolecular interaction between the JAK3 FERM domain and the γc chain of cytokine receptors may be functionally linked since alteration of the structure of the catalytic domain can disrupt the interaction with the receptor (Zhou et al., 2001). The relationship between FAK FERM domain mediated intra- and intermolecular interactions remains to be established. Since the intramolecular interaction is important for FAK signaling it is likely that the inter- and intramolecular interactions can occur at the same time. One intriguing possibility is that the

intramolecular interaction is promoted by intermolecular interactions, which would provide a mechanism for activation of FAK signaling by transmembrane proteins.

Previous hypotheses have speculated that regulation of FAK signaling occurs via several distinct mechanisms. One of these proposed mechanisms involves a hypothetical intramolecular inhibitory interaction involving the FERM domain of FAK (Schlaepfer and Hunter, 1998; Girault et al., 1999b; Toutant et al., 2002; Girault et al., 1999b). However, our current results suggest that a FERM domain mediated interaction promotes, rather than impairs, FAK signaling. Perhaps there are multiple FERM domain mediated interactions within FAK, one inhibitory and another stimulatory. Alternatively, FERM domain deletion mutants that activate FAK (Schlaepfer and Hunter, 1996; Toutant et al., 2002) may be defective for association with other unidentified FAK binding partners that function to inhibit FAK signaling. One of the most intriguing aspects of the interaction between the FERM domain and catalytic domain of FAK is the attenuation of signaling upon disruption of the interaction. This observation suggests that the F2 subdomain of FAK may be an effective target for therapeutic intervention in FAK signaling. Pharmacological disruption of the FAK FERM domain interaction with the catalytic domain might reduce phosphorylation of FAK and Src binding *in vivo* and impair aberrant cell motility or survival induced by FAK under pathological conditions.

EXPERIMENTAL PROCEDURES

Cells. Chicken embryo (CE) cells were prepared and maintained as described (Reynolds et al., 1989). The RCAS A avian replication competent retroviral vector encoding wild type FAK and CAKβ, FAK mutants and FAK/CAKβ chimeras have been described (Schaller and Sasaki, 1997; Schaller et al., 1994; Gabarra-Niecko et al., 2002; Dunty and Schaller, 2002). CE cells were transfected using Lipofectamine PLUS (Life Technologies, Gaithersburg, MD). For adhesion experiments, CE cells were trypsinized, held in suspension, and plated onto dishes coated with fibronectin (50 μg/ml) (Gabarra-Niecko et al., 2002; Shen and Schaller, 1999). In some experiments, cells were treated with 50 μM vanadate for 16 hours prior to lysis (Schaller and Sasaki, 1997). Rat-1 cells were cultured in DMEM + 10% fetal bovine serum. T47D cells were maintained in RPMI 1640 containing 10% fetal bovine serum and 0.2 units/ml insulin. Motility assays were performed as described, using 20 μg/ml of collagen I as the haptotactic stimulus (Gabarra-Niecko et al., 2002; Keely et al., 1995).

Protein Analysis. Cells were washed twice with phosphate-buffered saline (PBS) and lysed in Triton X-100 lysis buffer or Tx-RIPA containing protease and phosphatase inhibitors as described (Shen et al., 1998; Thomas et al., 1999). Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL). The KT3 monoclonal antibody (Covance, Princeton, NJ), the PY397 phosphorylation site specific antibody (Biosource International, Camarillo, CA), a Pyk2/CAKβ monoclonal antibody and the RC20 phosphotyrosine antibody (BD Biosciences, San Diego, CA) were purchased commercially. The BC4 polyclonal antiserum has been described (Shen and

Schaller, 1999; Schaller et al., 1992). The Fyn polyclonal antiserum was a generous gift from Dr. André Veillette (Institut de Recherches, Clinique de Montreal). Immunoprecipitations and Western blotting were preformed as previously described (Gabarra-Niecko et al., 2002). For co-immunoprecipitations, the immune complexes were washed with IPW buffer (20 mM Tris, pH 7.4, 10% glycerol, 50 mM NaCl, 0.2% Triton X-100). For *in vitro* kinase assays, immune complexes were washed with IPW buffer, PBS and kinase reaction buffer (20 mM PIPES, pH 7.2, 7.5 μM $MnCl_2$, 2.5 μM $MgCl_2$). The immune complexes were incubated in kinase reaction buffer containing 10 μCi γ[^{32}P]-ATP, 5 μM cold ATP and 50 μg of poly[Glu,Tyr] for the indicated times at room temperature. Kinase reactions were terminated with the addition of sample buffer and analyzed by SDS-PAGE and autoradiography. Quantitative analysis was performed using a Storm phosphorimager and ImageQuant software.

Homology Modeling of the FERM Domain of FAK. Homology modeling was performed in the Structural Bioinformatics Core facility at UNC-CH. The crystal structure of the FERM domain of moesin, (PDB ID = 1E5W) (Edwards and Keep, 2001), was used as the template for homology modeling the FERM domain of FAK. The initial alignment of the sequence of human FAK with the sequence of moesin was made using the 3D-PSSM fold recognition program and modified using the Clustal X multiple sequence alignment program (Kelley et al., 2000; Thompson et al., 1997). The Modeler module of the InsightII molecular modeling system (Accelrys Inc.) was used to create the model of the N-terminal domain of FAK. The sequence-structure compatibility of the model was evaluated using the Verify function of the Profiles-3D module of InsightII. The sequence alignment of FAK and moesin was modified in regions of the model with

low Profiles-3D/Verify scores. A new model was generated using the new alignment and the sequence-structure compatibility of the new model was evaluated. This process was repeated until the sequence-structure compatibility score could not be improved. The first two strands of the FAK FERM domain repeatedly received a low self-compatibility score and were deleted from the final model. The final model, which contains FAK residues 60-349, received a self-compatibility score of 119.9 out of a maximum expected score of 131.9. A score below 59 would indicate an incorrect structure. For comparison, the X-ray crystal structure of the FERM domain of moesin was analyzed using the Verify function of Profiles-3D. This structure received a self-compatibility score of 153 out of a maximum expected score of 158. This analysis suggests that this structure is a reasonably good homology model.

Recombinant proteins. The avian FAK or rat CAKβ sequences encoding residues 1-405 were amplified by PCR and subcloned into pGEX-KG in frame with the GST coding sequences (Guan and Dixon, 1991). All amplified sequences were analyzed by nucleotide sequencing at the UNC-CH Automated DNA Sequencing Facility on a model 377 DNA Sequencer (Perkin Elmer, Applied Biosystems Division) using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin Elmer, Applied Biosystems Division). The GST fusion proteins were induced as previously described (Thomas et al., 1999). For binding assays, cell lysates (1-2 mg) were pre-cleared with GST (25 μg) immobilized on glutathione agarose beads (Sigma) for 1 hour at 4°C. Pre-cleared lysates were incubated with GST fusion proteins (25μg) immobilized on glutathione agarose beads for 1 hour at 4°C with constant rocking, then washed 3 times in IPW buffer. Bound proteins were eluted with Laemmli

sample buffer and analyzed by Western blotting. For expression using baculovirus, the FAK cDNA was subcloned into the pFastBac vector, in frame with the His tag, and rescued into baculovirus using the Bac-to-Bac system (Life Technologies). Insect cells were infected with the virus encoding FAK, the cells lysed, and the His-tagged FAK purified by chromatography on a nickel column. To explore direct binding, 1.5 μg of purified recombinant FAK was incubated with 25 μg of GST fusion protein immobilized on glutathione beads, in 500 μl of Triton X-100 lysis buffer containing 1 mg/ml bovine serum albumin. The beads were washed three times with IPW buffer and the bound protein analyzed by Western blotting.

Molecular Biology. FAK mutants were engineered using pBS-FAK as a template (Schaller et al., 1992). Oligonucleotides were designed to substitute alanine residues for the amino acids targeted for mutagenesis and the mutations were created using the QuikChange mutagenesis kit (Stratagene, La Jolla CA). Mutants were analyzed by sequence analysis to verify the intended point mutations and that no unintended mutations were present. The N-terminal domains of the mutants (residues 1-405) were amplified and subcloned into pGEX-KG as described above. For expression in CE cells the mutant cDNAs were subcloned into RCAS A.

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REFERENCE LIST

Arold,S.T., Hoellerer,M.K., and Noble,M.E.M. (2002). The structural basis of localization and signaling by the focal adhesion targeting domain. Structure *10*, 319-327.

Calderwood,D.A., Yan,B., de Pereda,J.M., Alvarez,B.G., Fujioka,Y., Liddington,R.C., and Ginsberg,M.H. (2002). The phosphotyrosine binding-like domain of talin activates integrins. J. Biol. Chem. *277*, 21749-21758.

Cary,L.A., Chang,J.F., and Guan,J.L. (1996). Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. J. Cell Sci. *109 (Pt 7)*, 1787-1794.

Chen,R., Kim,O., Li,M., Xiong,X., Guan,J.L., Kung,H.J., Chen,H., Shimizu,Y., and Qiu,Y. (2001). Regulation of the PH-domain-containing tyrosine kinase Etk by focal adhesion kinase through the FERM domain. Nat. Cell Biol. *3*, 439-444.

Chishti,A.H., Kim,A.C., Marfatia,S.M., Lutchman,M., Hanspal,M., Jindal,H., Liu,S.C., Low,P.S., Rouleau,G.A., Mohandas,N., Chasis,J.A., Conboy,J.G., Gascard,P., Takakuwa,Y., Huang,S.C., Benz,E.J., Jr., Bretscher,A., Fehon,R.G., Gusella,J.F., Ramesh,V., Solomon,F., Marchesi,V.T., Tsukita,S., Tsukita,S., and Hoover,K.B. (1998). The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane [letter]. Trends Biochem. Sci. *23*, 281-282.

Dunty,J.M. and Schaller,M.D. (2002). The amino termini of FAK family kinases regulate substrate phosphorylation, localization, and cell morphology. J. Biol. Chem. *277*, 45644-45654.

Edwards,S.D. and Keep,N.H. (2001). The 2.7 A crystal structure of the activated FERM domain of moesin: an analysis of structural changes on activation. Biochemistry *40*, 7061-7068.

Frisch,S.M., Vuori,K., Ruoslahti,E., and Chan-Hui,P.Y. (1996). Control of adhesion-dependent cell survival by focal adhesion kinase. J. Cell Biol. *134*, 793-799.

Gabarra-Niecko,V., Keely,P.J., and Schaller,M.D. (2002). Characterization of an Activated Mutant of Focal Adhesion Kinase: SuperFAK. Biochem. J. *365*, 591-603.

Girault,J.A., Costa,A., Derkinderen,P., Studler,J.M., and Toutant,M. (1999b). FAK and PYK2/CAKbeta in the nervous system: a link between neuronal activity, plasticity and survival? Trends Neurosci. *22*, 257-263.

Girault,J.A., Labesse,G., Mornon,J.P., and Callebaut,I. (1999a). The N-termini of FAK and JAKs contain divergent band 4.1 domains. Trends Biochem. Sci. *24*, 54-57.

Golubovskaya,V., Beviglia,L., Xu,L.H., Earp,H.S., III, Craven,R., and Cance,W. (2002). Dual Inhibition of Focal Adhesion Kinase and Epidermal Growth Factor Receptor Pathways Cooperatively Induces Death Receptor-mediated Apoptosis in Human Breast Cancer Cells. J. Biol. Chem. *277*, 38978-38987.

Guan,K.L. and Dixon,J.E. (1991). Eukaryotic proteins expressed in Escherichia coli: and improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. *192*, 262-267.

Gusella,J.F., Ramesh,V., MacCollin,M., and Jacoby,L.B. (1999). Merlin: the neurofibromatosis 2 tumor suppressor. Biochim. Biophys. Acta *1423*, M29-M36.

Hamada,K., Shimizu,T., Matsui,T., Tsukita,S., and Hakoshima,T. (2000). Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. EMBO J. *19*, 4449-4462.

Hasson,T. (1999). Molecular motors: sensing a function for myosin-VIIa. Curr. Biol. *9*, R838-R841.

Hayashi,I., Vuori,K., and Liddington,R.C. (2002). The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. Nat. Struct. Biol. *9*, 101-106.

Hilkens,C.M., Is'harc,H., Lillemeier,B.F., Strobl,B., Bates,P.A., Behrmann,I., and Kerr,I.M. (2001). A region encompassing the FERM domain of Jak1 is necessary for binding to the cytokine receptor gp130. FEBS Lett. *505*, 87-91.

Ilic,D., Furuta,Y., Kanazawa,S., Takeda,N., Sobue,K., Nakatsuji,N., Nomura,S., Fujimoto,J., Okada,M., and Yamamoto,T. (1995). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature *377*, 539-544.

Keely,P.J., Fong,A.M., Zutter,M.M., and Santoro,S.A. (1995). Alteration of collagen-dependent adhesion, motility, and morphogenesis by the expression of antisense alpha 2 integrin mRNA in mammary cells. J. Cell Sci. *108 (Pt 2)*, 595-607.

Kelley,L.A., MacCallum,R.M., and Sternberg,M.J. (2000). Enhanced genome annotation using structural profiles in the program 3D- PSSM. J. Mol. Biol. *299*, 499-520.

LaJeunesse,D.R., McCartney,B.M., and Fehon,R.G. (1998). Structural analysis of Drosophila merlin reveals functional domains important for growth control and subcellular localization. J. Cell Biol. *141*, 1589-1599.

Liu,G., Guibao,C.D., and Zheng,J. (2002). Structural insight into the mechanisms of targeting and signaling of focal adhesion kinase. Mol. Cell Biol. *22*, 2751-2760.

Louvet-Vallee,S. (2000). ERM proteins: from cellular architecture to cell signaling. Biol. Cell *92*, 305-316.

Pearson,M.A., Reczek,D., Bretscher,A., and Karplus,P.A. (2000). Structure of the ERM protein moesin reveals the FERM domain fold masked by an extended actin binding tail domain. Cell *101*, 259-270.

Poullet,P., Gautreau,A., Kadare,G., Girault,J.A., Louvard,D., and Arpin,M. (2001). Ezrin interacts with focal adhesion kinase and induces its activation independently of cell-matrix adhesion. J. Biol. Chem. *276*, 37686-37691.

Reynolds,A.B., Roesel,D.J., Kanner,S.B., and Parsons,J.T. (1989). Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cells expressing oncogenic variants of the avian cellular src gene. Mol. Cell Biol. *9*, 629-638.

Schaller,M.D. (2001). Biochemical signals and biological responses elicited by the focal adhesion kinase. Biochim. Biophys. Acta *1540*, 1-21.

Schaller,M.D., Borgman,C.A., Cobb,B.S., Vines,R.R., Reynolds,A.B., and Parsons,J.T. (1992). pp125^{FAK} a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc. Natl. Acad. Sci. U. S. A *89*, 5192-5196.

Schaller,M.D., Hildebrand,J.D., Shannon,J.D., Fox,J.W., Vines,R.R., and Parsons,J.T. (1994). Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. Mol. Cell Biol. *14*, 1680-1688.

Schaller,M.D., Otey,C.A., Hildebrand,J.D., and Parsons,J.T. (1995). Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. J. Cell Biol. *130*, 1181-1187.

Schaller,M.D. and Parsons,J.T. (1995). pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high- affinity binding site for Crk. Mol. Cell Biol. *15*, 2635-2645.

Schaller,M.D. and Sasaki,T. (1997). Differential signaling by the focal adhesion kinase and cell adhesion kinase beta. J. Biol. Chem. *272*, 25319-25325.

Schlaepfer,D.D., Broome,M.A., and Hunter,T. (1997). Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. Mol. Cell Biol. *17*, 1702-1713.

Schlaepfer,D.D., Hauck,C.R., and Sieg,D.J. (1999). Signaling through focal adhesion kinase. Prog. Biophys. Mol. Biol. *71*, 435-478.

Schlaepfer,D.D. and Hunter,T. (1996). Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. Mol. Cell Biol. *16*, 5623-5633.

Schlaepfer,D.D. and Hunter,T. (1997). Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. J. Biol. Chem. *272*, 13189-13195.

Schlaepfer,D.D. and Hunter,T. (1998). Integrin signalling and tyrosine phosphorylation: just the FAKs? Trends Cell Biol. *8*, 151-157.

Shen,Y. and Schaller,M.D. (1999). Focal adhesion targeting: the critical determinant of FAK regulation and substrate phosphorylation. Mol. Biol. Cell *10*, 2507-2518.

Shen,Y., Schneider,G., Cloutier,J.F., Veillette,A., and Schaller,M.D. (1998). Direct association of protein-tyrosine phosphatase PTP-PEST with paxillin. J. Biol. Chem. *273*, 6474-6481.

Shimizu,T., Seto,A., Maita,N., Hamada,K., Tsukita,S., Tsukita,S., and Hakoshima,T. (2002). Structural basis for neurofibromatosis type 2. Crystal structure of the merlin FERM domain. J. Biol. Chem. *277*, 10332-10336.

Sieg,D.J., Hauck,C.R., Ilic,D., Klingbeil,C.K., Schaefer,E., Damsky,C.H., and Schlaepfer,D.D. (2000). FAK integrates growth-factor and integrin signals to promote cell migration. Nat. Cell Biol. *2*, 249-256.

Thomas,J.W., Cooley,M.A., Broome,J.M., Salgia,R., Griffin,J.D., Lombardo,C.R., and Schaller,M.D. (1999). The role of focal adhesion kinase binding in the regulation of tyrosine phosphorylation of paxillin. J. Biol. Chem. *274*, 36684-36692.

Thompson,J.D., Gibson,T.J., Plewniak,F., Jeanmougin,F., and Higgins,D.G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. *25*, 4876-4882.

Toutant,M., Costa,A., Studler,J.M., Kadare,G., Carnaud,M., and Girault,J.A. (2002). Alternative Splicing Controls the Mechanisms of FAK Autophosphorylation. Mol. Cell Biol. *22*, 7731-7743.

Ward,A.C., Touw,I., and Yoshimura,A. (2000). The Jak-Stat pathway in normal and perturbed hematopoiesis. Blood *95*, 19-29.

Zhou,Y.J., Chen,M., Cusack,N.A., Kimmel,L.H., Magnuson,K.S., Boyd,J.G., Lin,W., Roberts,J.L., Lengi,A., Buckley,R.H., Geahlen,R.L., Candotti,F., Gadina,M., Changelian,P.S., and O'Shea,J.J. (2001). Unexpected effects of FERM domain mutations on catalytic activity of Jak3: structural implication for Janus kinases. Mol. Cell *8*, 959-969.

FIGURE LEGENDS

Figure 1. The N-terminal domain of FAK associates with FAK *in vitro*. **A)** Epitope tagged FAK (lanes 1-4) or CAKβ (lanes 5-8) were expressed in CE cells. Cell lysates (1mg) were pre-cleared with GST bound to glutathione beads, then incubated with 25 μg of GST, GST-N-FAK or GST-N-CAKβ. Bound proteins were detected by Western blotting with KT3. Cell lysate (25 μg) was analyzed directly by Western blotting as a control (lanes 1 & 5). **B)** Rat-1 cells (lanes 1-4) or CE cells (lanes 5-8) were lysed, pre-cleared with GST, then incubated with GST, GST-N-FAK or GST-N-CAKβ. Endogenous FAK bound to the beads was detected by Western blotting with BC4. Lysate (25 μg) was directly blotted as a control (lanes 1 & 5). **C)** The blot in panel B was stripped and reprobed with a CAKβ monoclonal antibody to examine endogenous CAKβ binding to the GST fusion proteins. Note that CE cells express no endogenous CAKβ and these samples were excluded from the analysis. **D)** A fraction of the GST pulldowns performed on Rat 1 cell lysates (panels B & C) were analyzed by SDS-PAGE and Coomassie blue staining to demonstrate that equal amounts of each fusion protein was used in the analysis.

Figure 2. Association of the FERM domain with FAK/CAKβ chimeras and FAK mutants. **A)** Lysates of cells expressing the chimeras FFF (lanes 1-4), CFC (lanes 5-8), CCC (lanes 9-12), and FCF (lanes 13-16) were incubated with GST fusion proteins immobilized to glutathione agarose beads. Chimeric proteins that bound to GST-N-FAK,

GST-N-CAKβ or GST alone were detected by Western blotting with KT3. Lysate was directly analyzed as a control (lanes 1, 5, 9 & 13). **B)** Lysates of CE cells expressing FAK (lanes 1-3), FAKY397F (lanes 4 & 5) or FAKK454M (lanes 6 & 7) were incubated with GST-N-FAK, and bound FAK was detected by Western blotting. Lysates were directly analyzed by Western blotting as a control (lanes 1, 4 & 6). As a negative control for FAK binding, GST was used.

Figure 3. Model of the FERM domain of FAK. A) The backbone of the model of the FERM domain of FAK is shown. This model contains FAK residues 60-349. The ubiquitin-like subdomain (F1) , acyl CoA binding protein-like subdomain (F2) and PH/PTB/EVH-like subdomain (F3) are indicated. **B)** The surface of the model of the FERM domain is shown. In the top panel, electrostatic potential is indicated colorimetrically with red denoting negative potential and blue indicating positive potential. In the middle panel, the surface of the FERM domain is shown with residues that are identical from Drosophila to human (green) and highly conserved residues (black) are indicated. In the bottom panel, the surface of the FERM domain is shown with residues that are identical between FAK and CAKβ colored yellow. The circled region indicates a highly conserved basic patch at the tip of the F2 subdomain.

Figure 4. Point mutations in the FERM domain disrupt the interaction with FAK. **A)** Lysates of CE cells overexpressing epitope tagged FAK were pre-cleared with GST, then incubated with GST, GST-N-FAK, GST-KAKTLR or GST-KK. Bound FAK was detected by Western blotting with KT3. Lysate (25 μg) was analyzed as a control (lane

1). **B)** As a loading control, a fraction of each pulldown from A was analyzed by SDS-PAGE and Coomassie blue staining. **C)** Twenty-five μg of GST, GST-N-FAK or GST-KAKTLR immobilized to glutathione agarose beads was incubated with purified recombinant FAK (1.5 μg) in Triton X-100 lysis buffer containing 1 mg/ml BSA. After washing, bound recombinant FAK was detected by Western blotting. **D)** A fraction of each pulldown from C was analyzed by SDS-PAGE and Coomassie blue staining.

Figure 5. KAKTLR is defective for tyrosine phosphorylation *in vivo*. A) In the upper panel, twenty-five μg of cell lysate from control transfected CE cells, FAK or KAKTLR expressing CE cells was analyzed by Western blotting for phosphotyrosine. The positions of molecular weight markers are indicated on the left. The blot was stripped and reprobed for FAK (lower panel). **B)** FAK was immunoprecipitated from lysates of CE cells containing empty vector or expressing FAK or KAKTLR. Immune complexes were Western blotted for phosphotyrosine (top panel) then stripped and reprobed for FAK (bottom panel). **C)** Twenty-five μg of cell lysate from control transfected CE cells, FAK or KAKTLR expressing CE cells was analyzed by Western blotting with a phospho-specific antibody recognizing FAK when phosphorylated on Y397. Lysate was also blotted for FAK to demonstrate equal expression of protein (bottom panel).

Figure 6. KAKTLR is defective for adhesion dependent tyrosine phosphorylation. CE cells expressing wild type FAK (lanes 1-5) or KAKTLR (lanes 6-10) were held in suspension or plated onto fibronectin for the indicated times. Tyrosine phosphorylation

in subconfluent cells in culture was also examined (lanes 1 & 6). FAK/KAKTLR was immunoprecipitated and analyzed by Western blotting for phosphotyrosine (panel A, top). Tyrosine phosphorylation at 397 was examined by Western blotting whole cell lysates with PY397 (panel B, top). As a loading control, these blots were stripped and reprobed using BC4 (panels A & B, bottom).

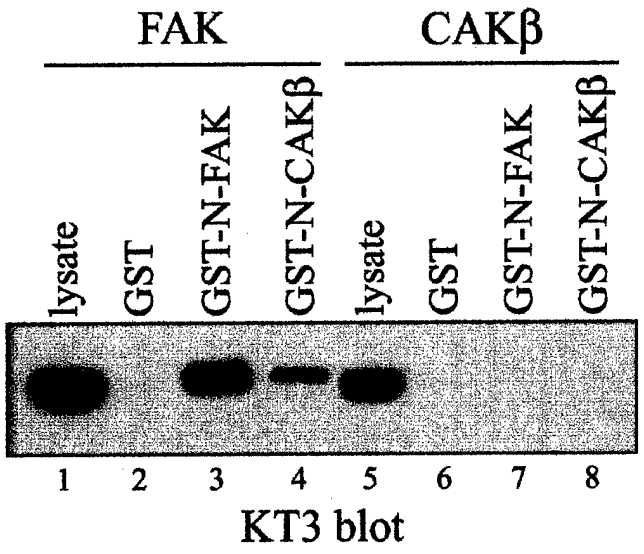
Figure 7. KAKTLR is catalytically active, but defective for Fyn binding. A) Endogenous FAK (lanes 1 & 2) and exogenously expressed wild type FAK (lanes 3 & 4) or KAKTLR (lanes 5 & 6) were immunoprecipitated from CE cell lysates, and the immune complexes incubated in an *in vitro* kinase assay containing poly[Glu,Tyr] for 5 or 10 minutes (top panel). A fraction of each immune complex was also blotted for FAK to verify equal recovery of wild type FAK (lane 2) and KAKTLR (lane 3)(bottom panel). **B)** The results from 5 experiments were quantified by phosphorimager analysis. Phosphorylation of substrate following 5 minutes incubation with FAK was arbitrarily set as 1. The error bars denote standard error. **C)** CE lysates containing empty vector or expressing FAK or KAKTLR were used for immunoprecipitations with a Fyn polyclonal antiserum. The immune complexes were blotted with BC4 to detect coimmunoprecipitated FAK (top panel), then the blot was stripped and reprobed for Fyn (bottom panel) to demonstrate equal recovery of Fyn in the immunoprecipitations.

Figure 8. KAKTLR is defective for signaling *in vivo*. A) Lysates of CE cells expressing empty RCAS A (lanes 1 & 2), FAK (lanes 3 & 4) or KAKTLR (lanes 5 & 6) were analyzed by Western blotting for phosphotyrosine. Prior to lysis cells were either

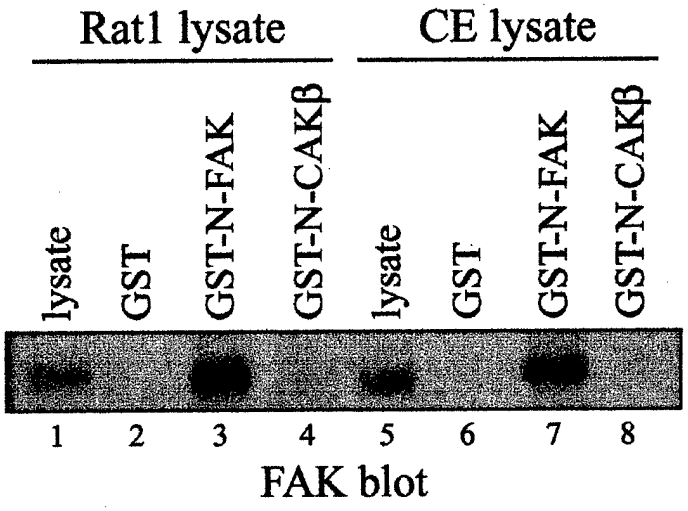
left untreated or treated with 50 μM vanadate for 16 hours. **B)** T47D/tva cells infected with empty RCAS A or expressing FAK or KAKTLR were assessed for haptotactic motility in response to collagen I. The average of 5 experiments, each performed in triplicate, is shown. Error bars denote standard error.

Figure 9. Models of FERM domain regulation of FAK signaling. A) An intramolecular interaction between the FERM domain and catalytic domain (red asterisk) promotes a conformation of the intervening loop that allows for tyrosine phosphorylation (PY) and Src kinase binding. Alternatively, an intermolecular FERM domain/catalytic domain interaction (red asterisks) may alter the conformation around tyrosine 397 promoting phosphorylation (PY) and Src kinase binding (**B**). The KAKTLR mutation disrupts this interaction and the loop between the FERM domain and catalytic domain cannot be oriented for optimal phosphorylation and Src kinase binding (**C**).

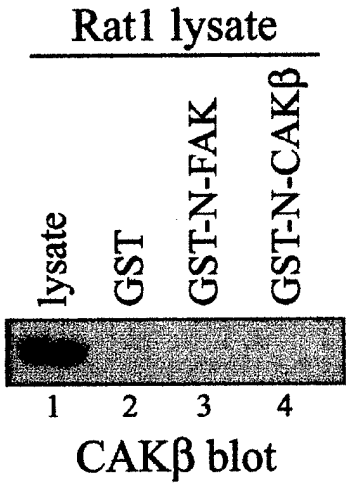
A.


FAK
CAKβ
lysate
GST
GST-N-FAK
GST-N-CAKβ
lysate
GST
GST-N-FAK
GST-N-CAKβ
1 2 3 4 5 6 7 8
KT3 blot

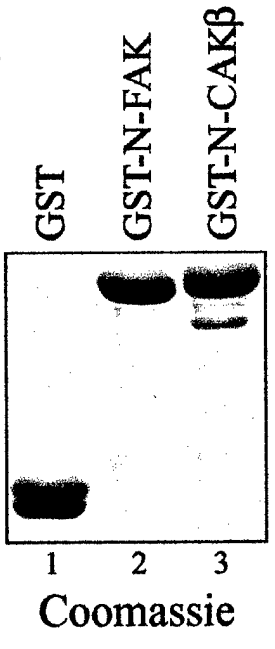
B.


Rat1 lysate
CE lysate
lysate
GST
GST-N-FAK
GST-N-CAKβ
lysate
GST
GST-N-FAK
GST-N-CAKβ
1 2 3 4 5 6 7 8
FAK blot

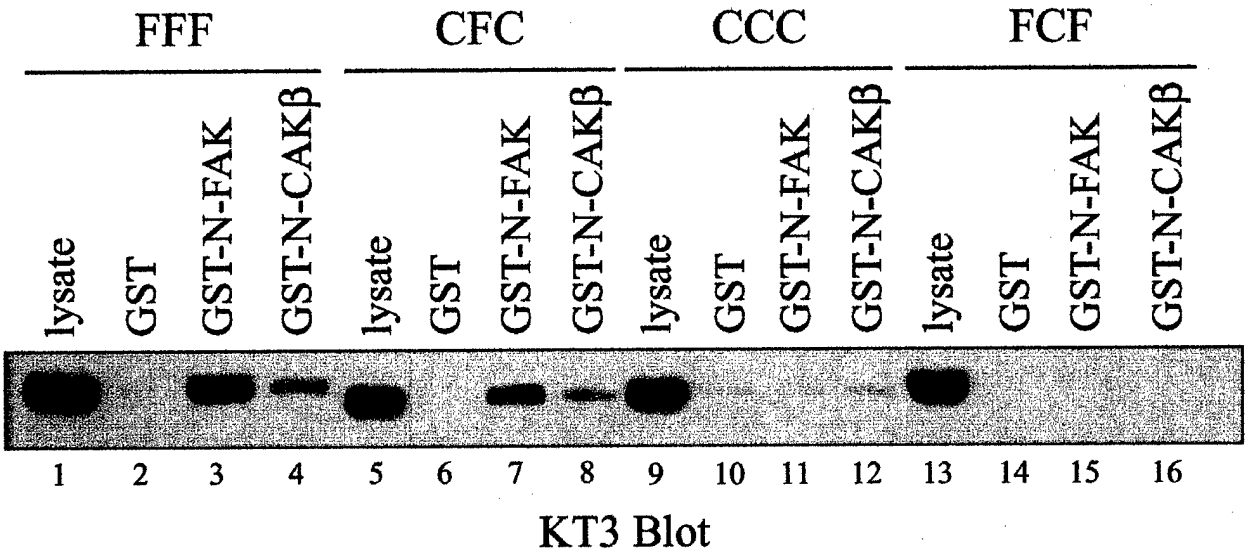
C.


Rat1 lysate
lysate
GST
GST-N-FAK
GST-N-CAKβ
1 2 3 4
CAKβ blot

D.

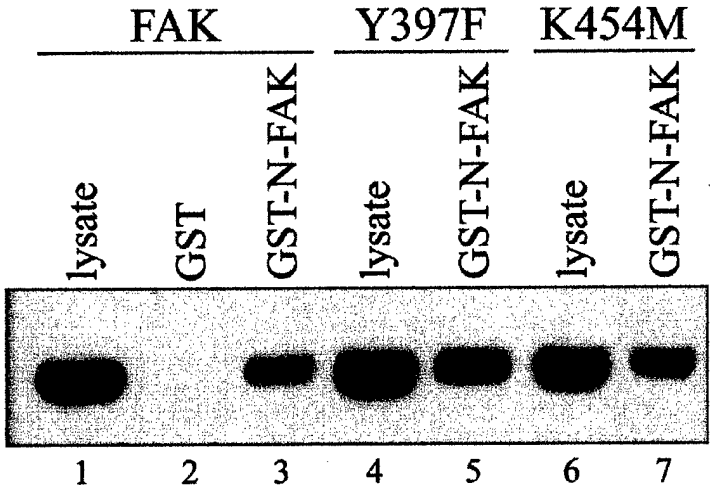

GST
GST-N-FAK
GST-N-CAKβ
1 2 3
Coomassie

A.

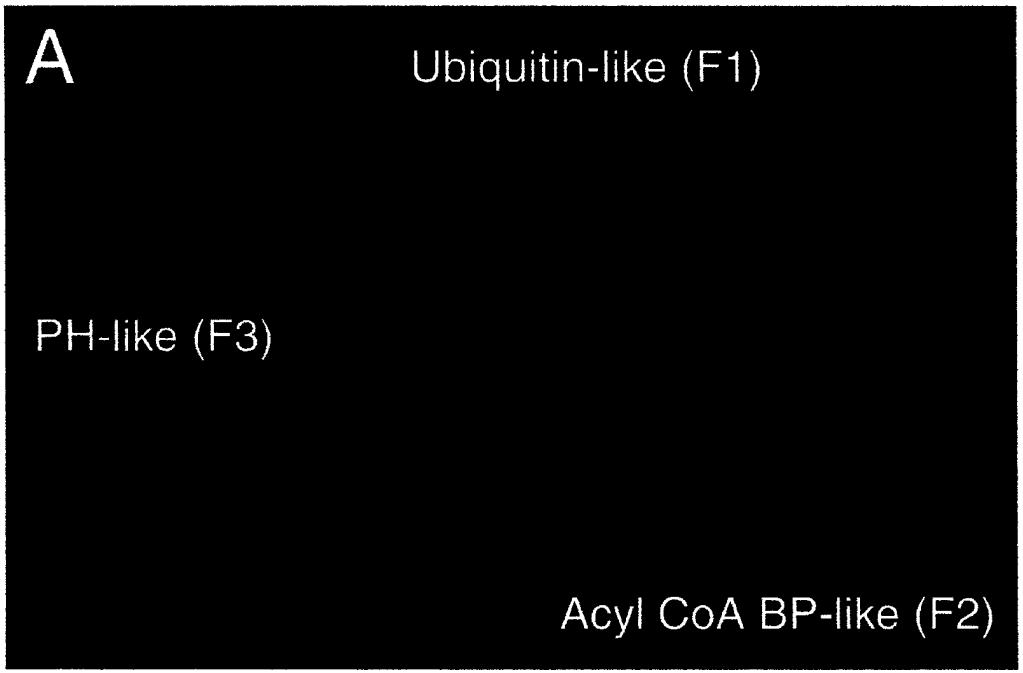


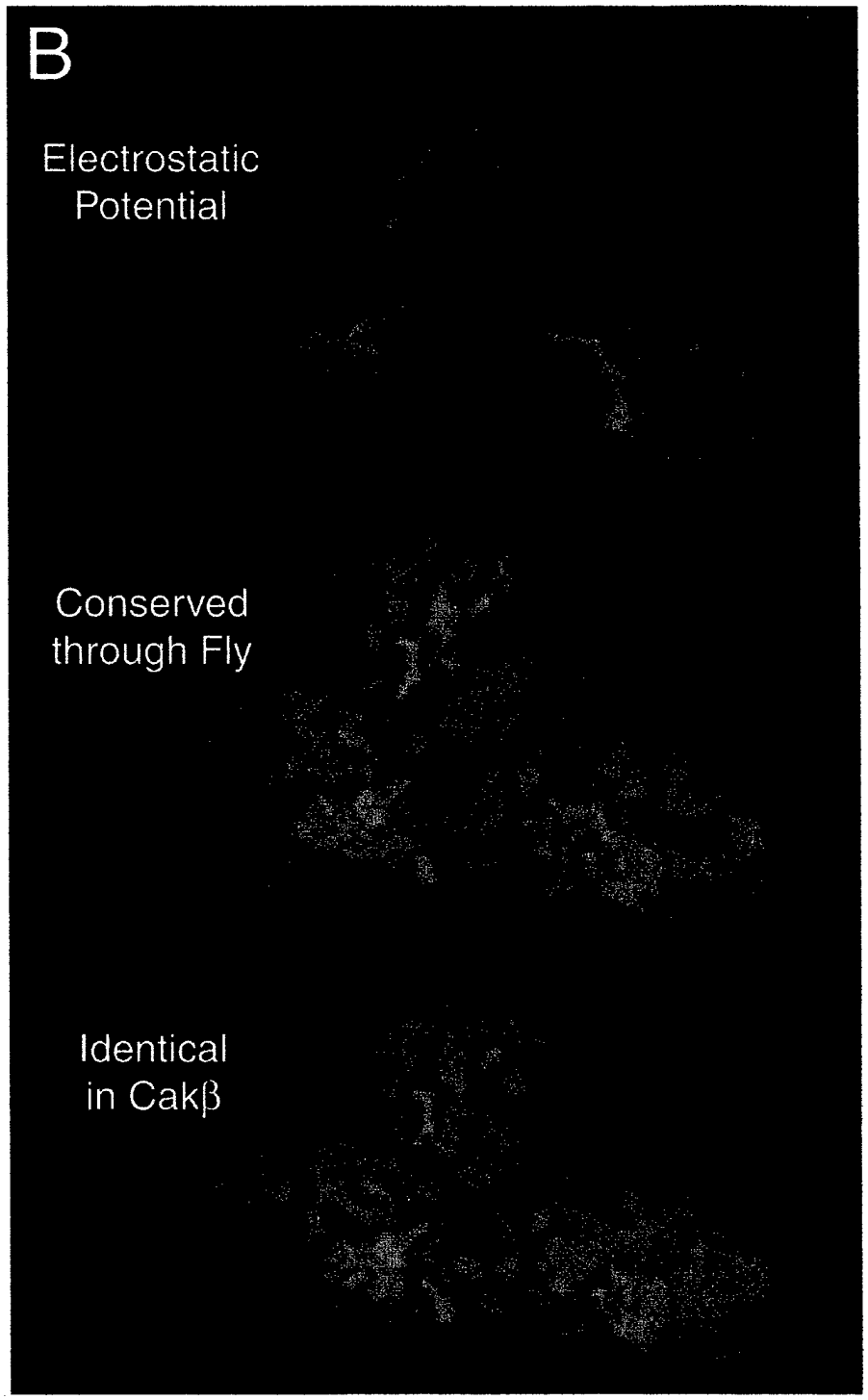
KT3 Blot

B.

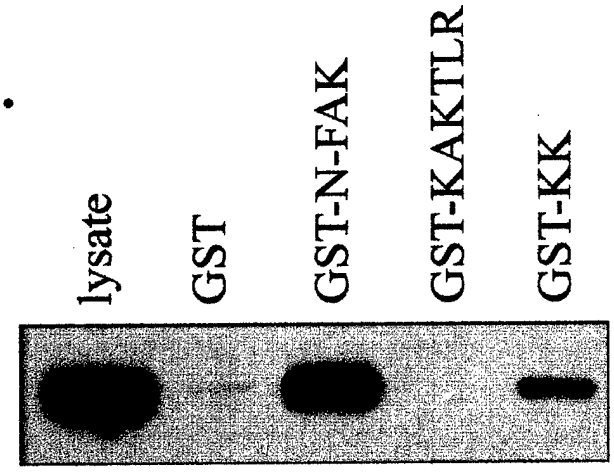


FAK Blot


A
Ubiquitin-like (F1)
PH-like (F3)
Acyl CoA BP-like (F2)

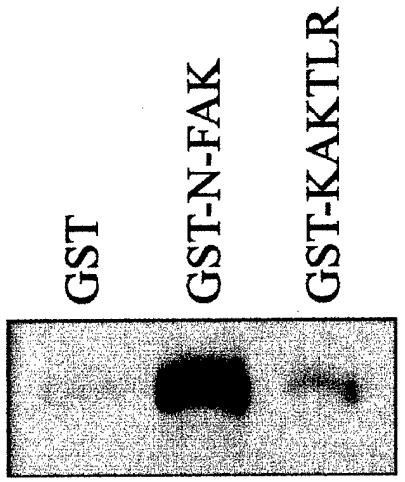

B
Electrostatic Potential
Conserved through Fly
Identical in Cakβ

A.



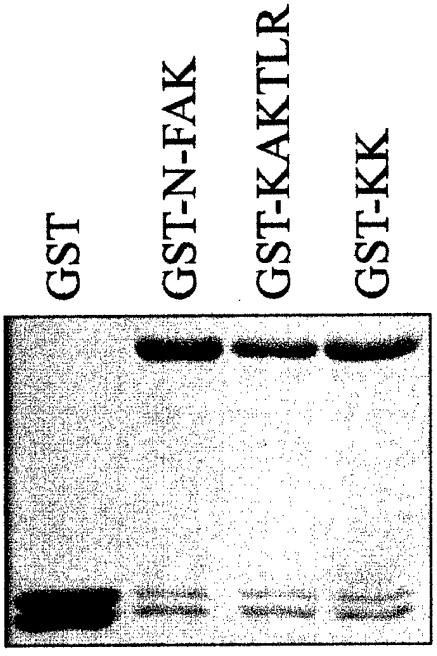
KT3 blot

C.



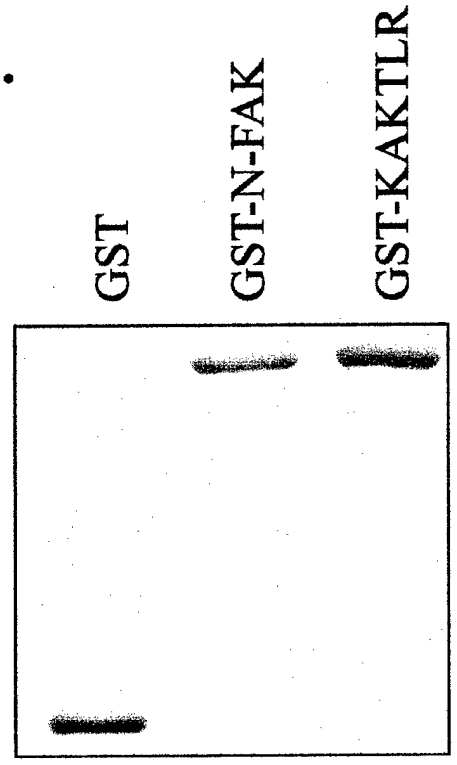
FAK blot

B.

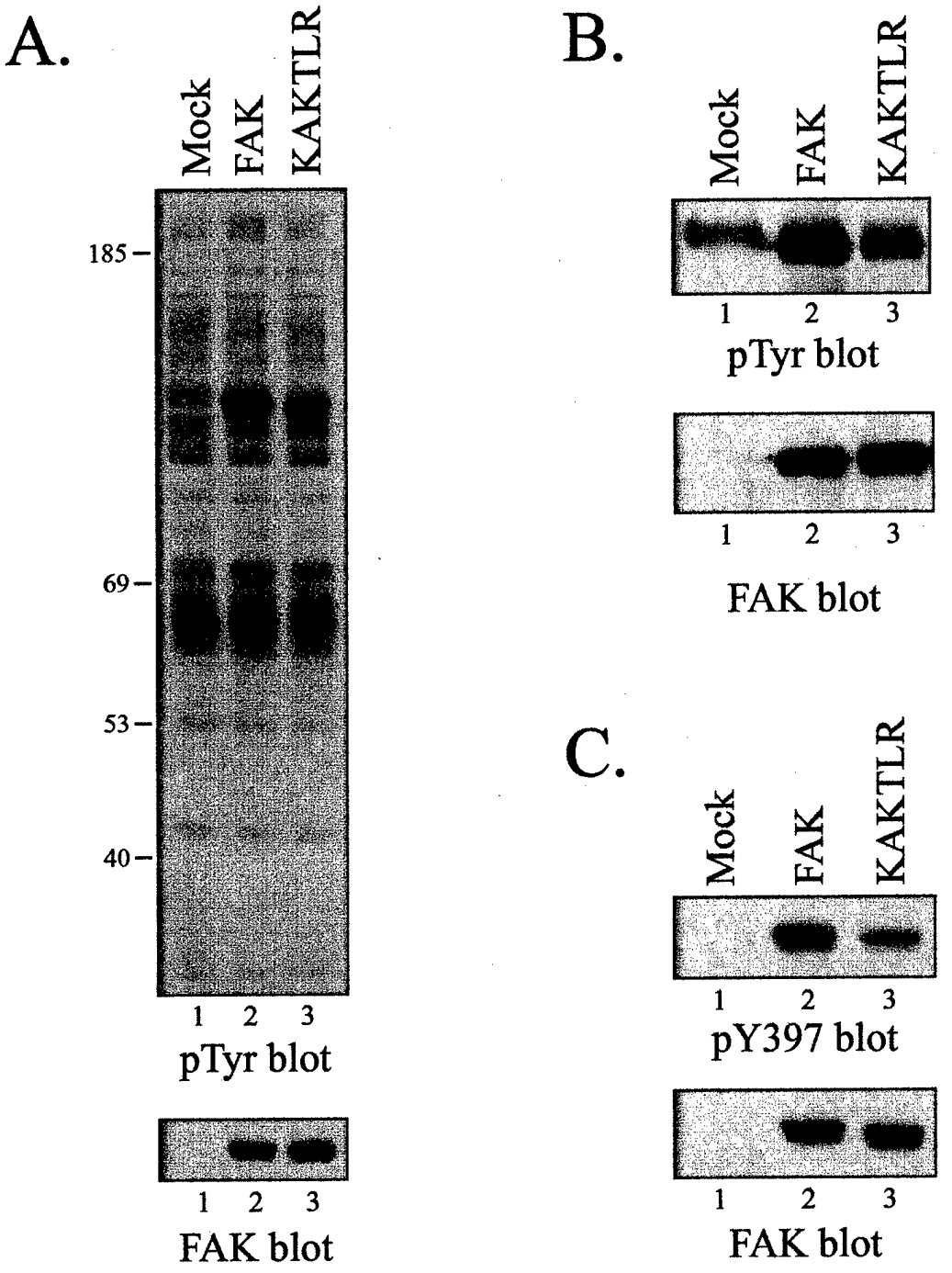


Coomassie

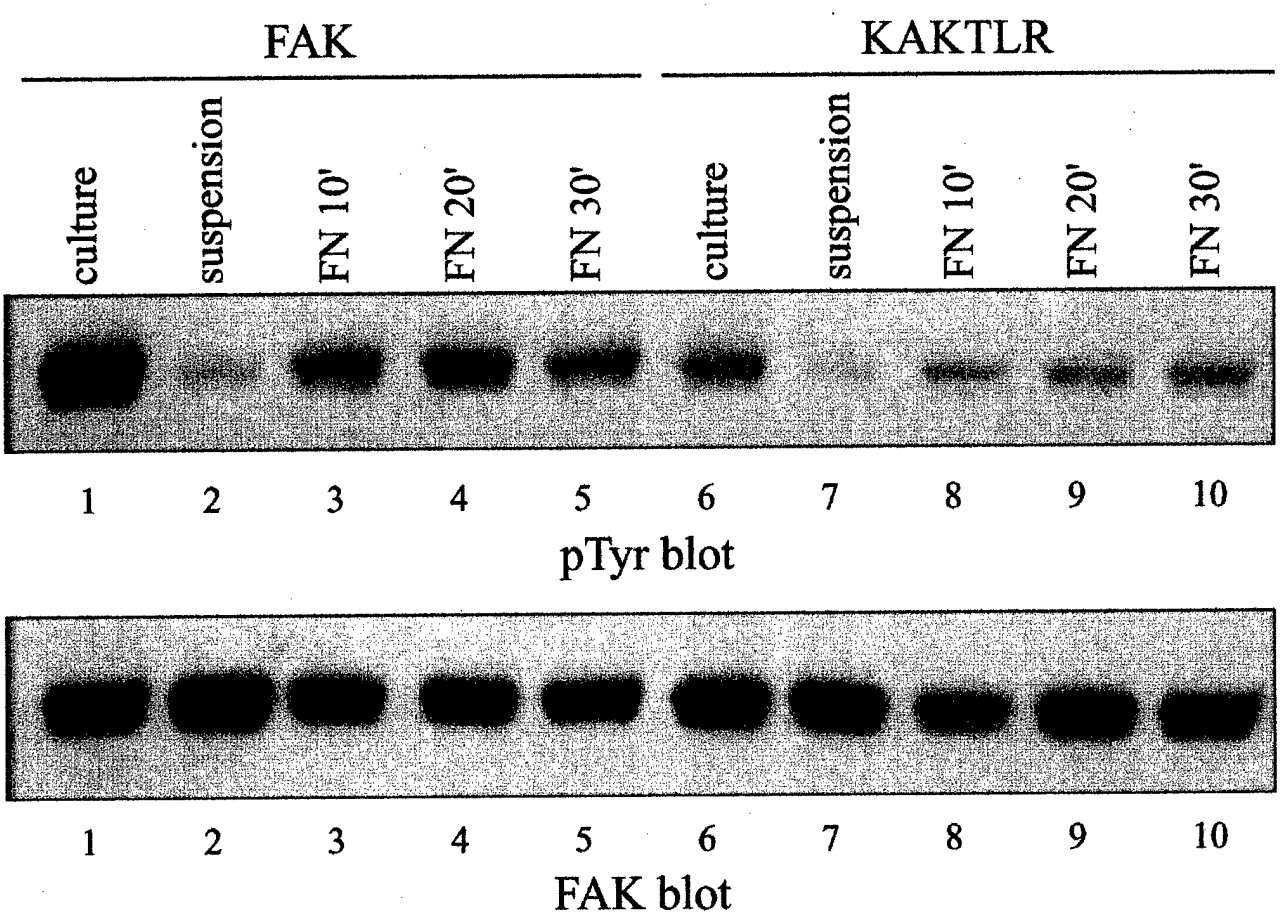
D.



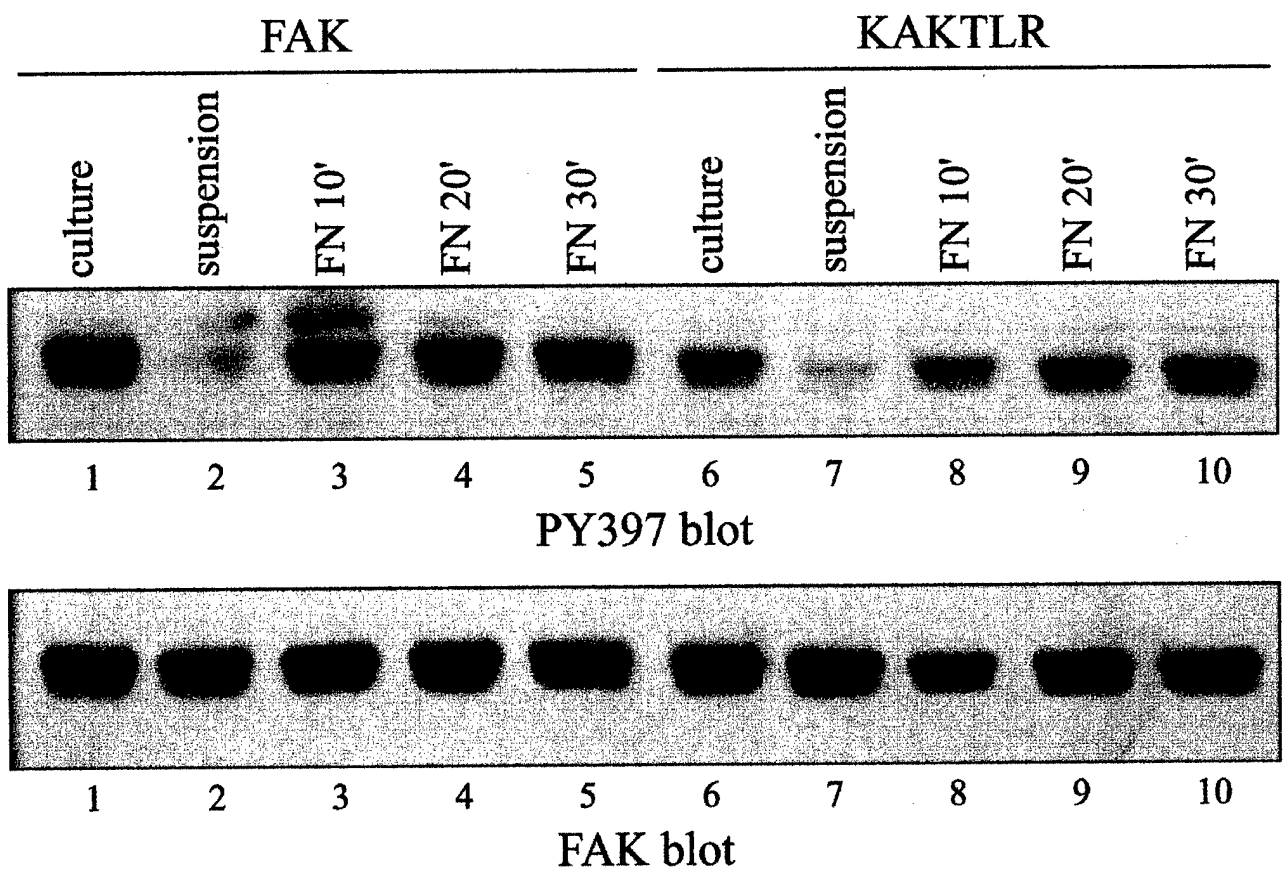
Coomassie


A.
Mock
FAK
KAKTLR
185
69
53
40
1 2 3
pTyr blot
1 2 3
FAK blot
B.
Mock
FAK
KAKTLR
1 2 3
pTyr blot
1 2 3
FAK blot
C.
Mock
FAK
KAKTLR
1 2 3
pY397 blot
1 2 3
FAK blot

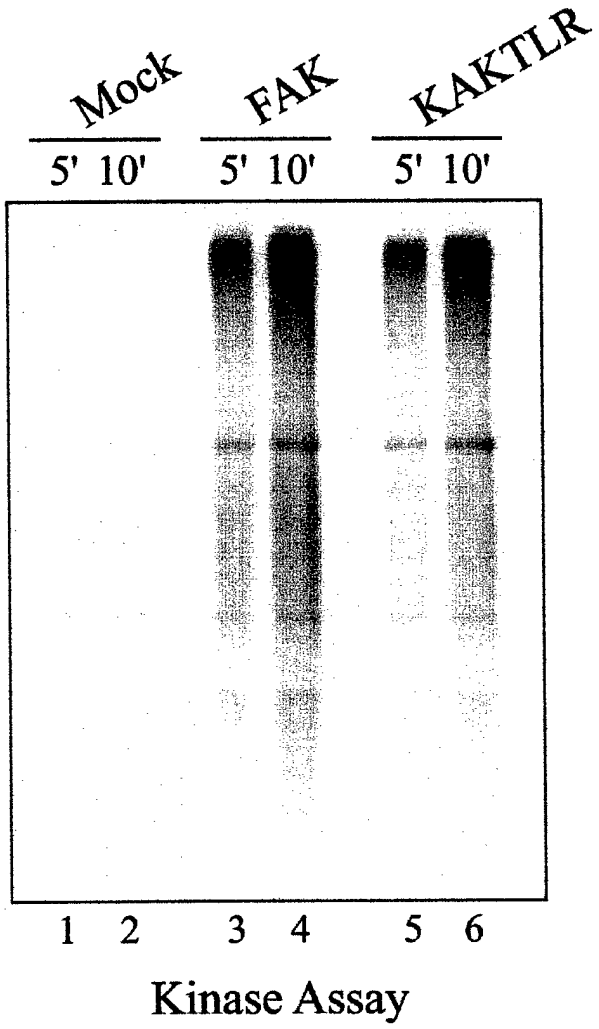
A.


FAK
KAKTLR
culture
suspension
FN 10'
FN 20'
FN 30'
culture
suspension
FN 10'
FN 20'
FN 30'
1 2 3 4 5 6 7 8 9 10
pTyr blot
1 2 3 4 5 6 7 8 9 10
FAK blot

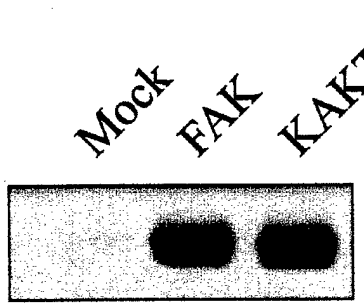
B.


FAK
KAKTLR
culture
suspension
FN 10'
FN 20'
FN 30'
culture
suspension
FN 10'
FN 20'
FN 30'
1 2 3 4 5 6 7 8 9 10
PY397 blot
1 2 3 4 5 6 7 8 9 10
FAK blot

A.

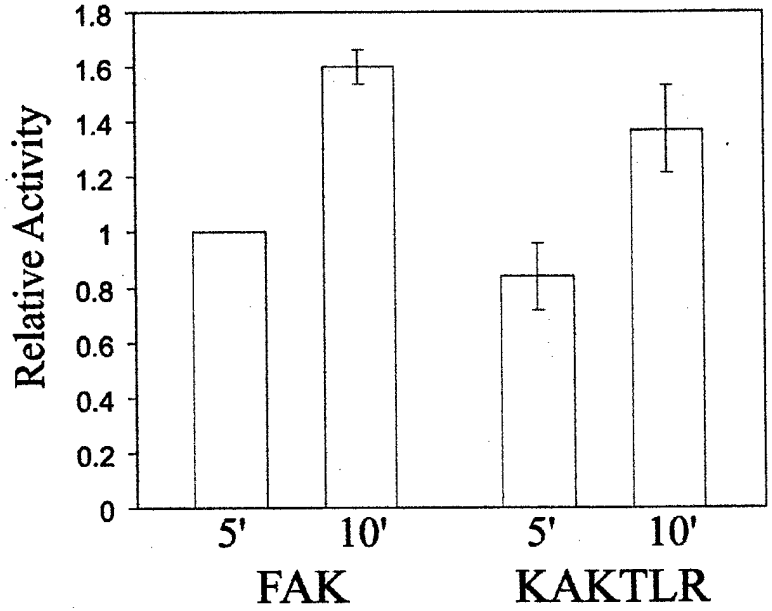


Kinase Assay

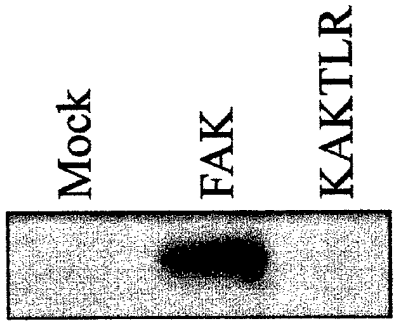


FAK blot

B.



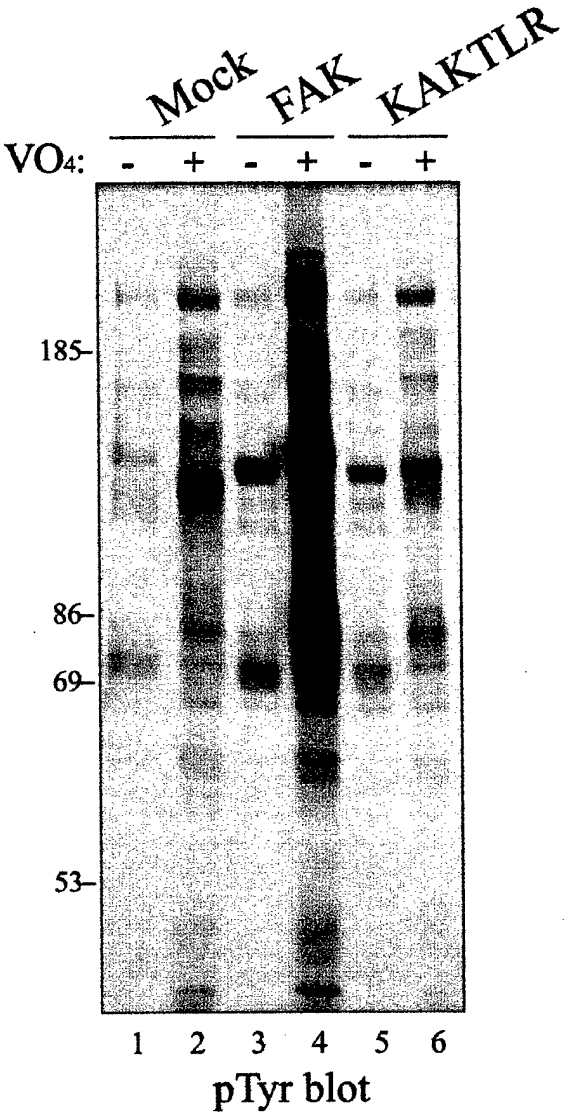
C.



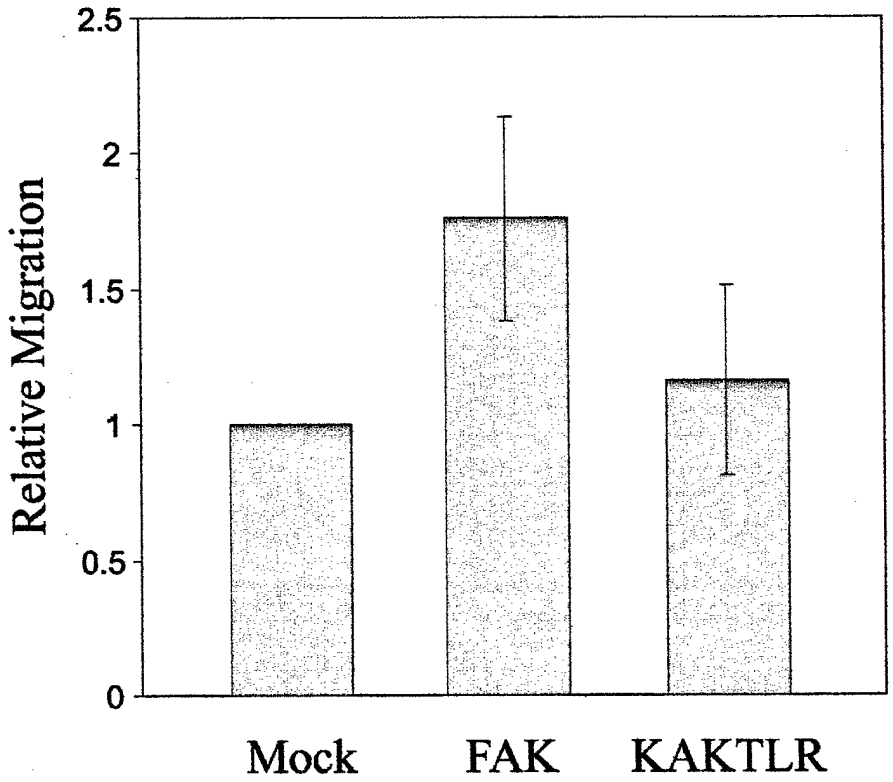
FAK blot

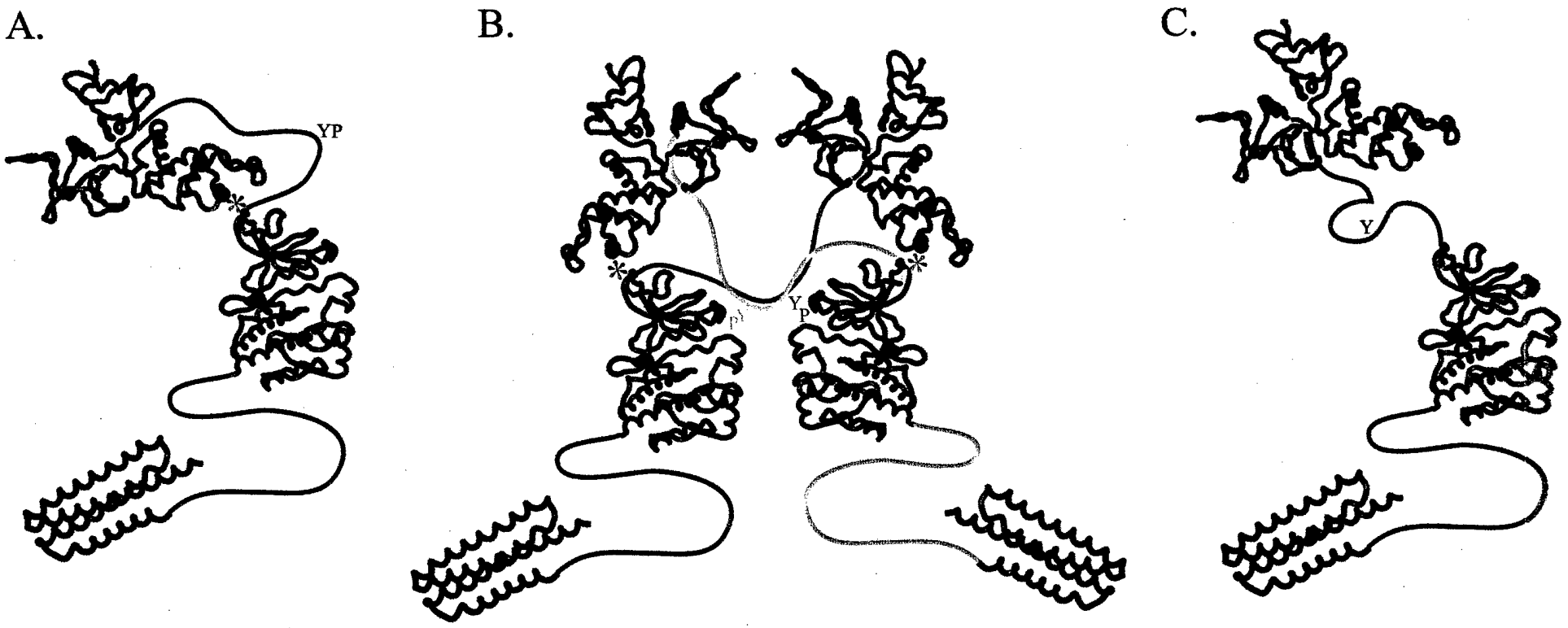
Fyn blot

A.


Mock
FAK
KAKTLR
VO4: - + - + - +
185-
86-
69-
53-
1 2 3 4 5 6
pTyr blot

B.


Relative Migration
2.5
2
1.5
1
0.5
0
Mock
FAK
KAKTLR


A.
YP
B.
C.
Y

APPENDIX E

Manuscript in Progress.

"The Role of FAK Signaling in Tumorigenesis of Breast Cancer Epithelial Cells"

THE ROLE OF FAK SIGNALING IN TUMORIGENESIS OF BREAST EPITHELIAL CELLS

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Department of Cell and Developmental Biology

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Introduction

Cells interact, sense and respond to their environment by transmitting information from the outside of the cell in the form of intracellular signals that act upon cellular processes. Focal adhesions (FA) are sites of close contact between the cell and the extracellular matrix to which it attaches. The integrins, heterodimeric extracellular matrix receptors, localize to FAs where they structurally connect the cell to the outside environment. Furthermore, integrin engagement to extracellular matrix ligands allows for recruitment of signaling proteins, including the focal adhesion kinase (FAK), to the FA. These signaling proteins can sense information about the cellular environment via the integrins and respond by regulating the appropriate processes inside the cell. Thus, FAs are important structural and signaling components of the cell.

Focal Adhesion Kinase (FAK) is a cytoplasmic tyrosine kinase that localizes to focal adhesions where it mediates signaling responses to the cellular environment detected by the integrins (1,178,234). It has unique domain structure consisting of a central catalytic domain, flanked by large carboxyl and amino terminal domains. A focal adhesion targeting sequence in the carboxy terminal domain of the protein is responsible for the subcellular localization of FAK (37). Although FAK does not contain any SH2 or SH3 modules, it contains proline rich regions and sites of tyrosine phosphorylation that recruit proteins for interaction. Upon activation by adhesion or treatment with soluble factors (1,178,234), FAK autophosphorylates on Y397 creating a binding site for the SH2 domain of Src kinases, Phosphotidylinositol 3-kinase (PI3K), Phospholipase C-γ-1, growth-factor-receptor-bound protein 7 (Grb7), and possibly Shc (24,141,231,235,236). Upon binding, Src can further phosphorylate tyrosines Y576 and Y577 in the catalytic domain of FAK leading to full

activation of the kinase (26). Src also phosphorylates Y925 located in the carboxy domain of FAK, creating a binding site for the SH2 domain of Grb2, and thus linking FAK to the MAPK cascade (25,26,30). In addition to SH2-binding sites, FAK also contains proline-rich regions that serve as docking sites for SH3-containing proteins, including the crk-associated protein, p130cas, and GRAF (33,34,237,238). The carboxy terminus also contains binding sites for paxillin and talin that overlap with the focal adhesion targeting sequence (36,38). FAK can thus recruit a variety of signaling proteins through which it can regulate cellular processes.

FAK has been implicated in the regulation of a variety of cellular processes, including cell proliferation, cell survival and cell motility. FAK is thought to be a positive mediator of the G1-S transition of the cell cycle by controlling the levels of cyclin D and cyclin-dependent kinase inhibitor p21 (23,239). In addition to cell proliferation, FAK is thought to mediate cellular survival signals. The inhibition of endogenous FAK causes cells to undergo apoptosis (20,180). Conversely, constitutively activated FAK can rescue cells grown in suspension from undergoing anoikis (21). PI 3-kinase, p130cas and Grb2 have been proposed as mediators of the FAK survival signal during UV and hydrogen peroxide-treatment (240,241). FAK has also been shown to be a regulator of cell motility. FAK null cells or cells expressing FRNK, a naturally occurring FAK dominant negative variant, show decreased motility (5,6,63). Conversely, overexpression of FAK increases the motility of Chinese hamster ovary cells and T47D cells (3,137). The autophosphorylation site on FAK, Src and PI3K binding, as well as p130cas have all been implicated in FAK-mediated regulation of motility (35,137,141).

FAK was first identified as a highly phosphorylated protein in cells transformed by the src oncogene (7) and eventually FAK was identified as a binding partner and substrate of Src (24,26,29). Furthermore, src has been found to be activated in a variety of tumors (151-157). Integrin expression can influence the tumorigenic properties of cells (158-162). In view of the tight link of FAK with Src and integrin-signaling, it is feasible that FAK signaling *per se* might be an important regulator of cancer progression. In support of this hypothesis, FAK is overexpressed in a variety of cancers as early as pre-invasive lesions suggesting an active or permissive role for the appearance and progression of cancer (2). Most importantly, FAK regulates normal cellular processes that when aberrant can lead to typical phenotypes associated with cancer. This suggests potential mechanisms through which uncontrolled FAK signaling could influence cancer pathogenesis. The accumulating evidence points at FAK as a potentially important player in cancer (2).

In this study, we used a cell model system to study the role that FAK signaling may play in the acquisition of cancer phenotypes in breast cancer epithelial cells. FAK signaling was manipulated in the breast cancer epithelial cells, T47D and changes in cancerous phenotypes were monitored. In order to increase FAK signaling, wild type FAK or a hyperactive mutant of FAK, SuperFAK, was expressed in T47D cells (3). Increased FAK signaling upon SuperFAK expression increased *in vivo* growth of T47D tumors. Conversely, in order to inhibit FAK signaling, FRNK, a naturally occurring dominant negative of FAK, was introduced in T47D cells (5,6). A significant decrease in the adhesion independent growth in soft agar of FRNK expressing T47D cells was observed. Interestingly, neither SuperFAK nor FRNK expression seem to affect the cell growth nor the suspension survival

of the breast cancer epithelial cells. Thus, FAK signaling plays a regulatory role in cancer pathogenesis suggesting FAK as a potential therapeutic target in cancer.

Material and Methods

Cell Culture

Chicken embryo (CE) fibroblasts (242) were harvested from 9-day-old embryos and grown in Dulbecco's modified Eagles's medium (Gibco/BRL, Rockville, MD) supplemented with 4% fetal bovine serum (Gibco/BRL, Rockville, MD), 1% chicken serum (Sigma, St. Louis, MO) as described (223). CE fibroblasts were maintained in a 39° C and 5% CO_2 incubator.

T47D breast epithelial cells stably expressing the avian retroviral receptor, Tva800 (T47D/Tva) were maintained in RPMI 1640 (Gibco/BRL, Rockville, MD) medium supplemented with 10% fetal bovine serum (Gibco/BRL, Rockville, MD), 0.2 U/ml insulin (Gibco/BRL, Rockville, MD), penicillin, streptomycin, genamycin and kanamycin (Sigma, St. Louis, MO) as described (3). T47D were maintained in a 37°C and 5% CO_2 incubator.

Transfection and Infection

CE cells were transfected with RCAS plasmid DNA using the LipofectAMINE PLUS™ reagent (Invitrogen, Carlsbad, CA) following the manufacturer's recommended protocol. Seven days after transfection, cells were lysed and expression was analyzed.

Viral avian retrovirus stocks were made from subconfluent cultures of CE cells 10 days after transfection. The culture medium was removed, 4 mls of fresh culture medium was added, and the cells were incubated overnight. The culture medium was collected, cells and debris were pelleted by centrifugation, and virus-containing supernatants were aliquoted and

stored at -70° C. Upon passaging T47D/Tva cells, 1 ml of virus stock was added to the T47D/Tva cultures. Ten to fourteen days after infection, cells were lysed and expression was analyzed.

Antibodies

Polyclonal FAK Ab, BC4, was a generous gift from Dr. J.T. Parsons. Monoclonal paxillin and p130cas were purchased commercially (Tranduction Labs, Lexington, KY). Horseradish conjugated to protein A or anti-mouse IgG was used to recognize the primary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Horseradish conjugated RC20, a recombinant derivative of PY20 mAb, were used to detect phosphotyrosine (Transduction Labs, Lexington, KY).

Protein Analysis

Cells were lysed in modified radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl pH 7.3, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate) containing protease and phosphatase inhibitors (50 ug/ml leupeptin, 1.5 mM PMSF, 0.5% aprotinin, 1.5 mM Na orthovanadate) for 10 min on ice (86). Lysates were clarified by centrifugation at 4° C. The protein concentration of lysates' were determined using the bicinchonic acid assay kit (Pierce, Rockford, IL).

For immunoprecipitations, 0.3-1 mg of cell lysate was incubated with primary antibody on ice for 1 hr. The polyclonal FAK antibody BC4, and the monoclonal paxillin and $p130^{cas}$ antibodies were used for immunoprecipitations. Immune complexes were precipitated with protein A sepharose beads (Sigma, St. Louis, MO), or rabbit anti-mouse IgG (Jackson

Immunoresearch Laboratories Inc., West Grove, PA) pre-bound to protein A sepharose beads (Sigma, St. Louis, MO) at 4° C for 1 hr. The immune complexes were then washed twice with modified RIPA buffer, and twice with Tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl, pH 7.0). Immune complexes were denatured and dissociated from beads by boiling in Laemmli sample buffer (226). The samples were then resolved by SDS-PAGE on an 8% gel, and analyzed by Western blotting. Nitrocellulose membranes were blocked with TBS-T (10 mM Tris, 150 mM NaCl, pH 7.0, containing 0.1% Tween 20) containing 5% w/v powdered milk or with TBS-T alone when using the RC20 phosphotyrosine antibody for 1 hr at room temperature. Membranes were incubated with primary antibody in blocking solution for 1 hr at room temperature. The antibodies described above were used for Western blotting. Primary antibodies were detected using horseradish peroxidase conjugated to protein A or anti-mouse IgG and Enhanced Chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

In vitro Kinase Assays

For *in vitro* kinase reactions, FAK immune complexes were washed twice in modified RIPA buffer, twice with TBS and once with kinase reaction buffer (20 mM PIPES pH 7.2, 3 mM $MnCl_2$ and $MgCl_2$). The immune complexes were then incubated in kinase buffer containing 10 μCi γ^{32}P-ATP (DupontNEN, Wilmington, DE) at room temperature for the times indicated. The kinase reactions were stopped by boiling in Laemmli sample buffer (226). The reactions were subjected to SDS-PAGE. The gels were fixed in 7% acetic acid and 20% methanol and dried. ^{32}P incorporation was visualized by autoradiography and phosphorimager analysis using the Storm860 (Molecular Dynamics, Sunnyvale, CA).

Motility

Motility assays were performed as described previously (228). The underside of 12 mm transwell chambers with a 12 μm pore polycarbonate membrane (Costar, Cambridge, MA) were coated with 0.6 ml of 40 μg/ml rat tail collagen I (Collaborative Biomedical Products, Bedford, MA) for 6 hrs at 37° C. The lower chamber was washed twice and filled with serum free RPMI1640 medium. T47D cells were trypsinized, counted and resuspended in RPMI1640 medium supplemented with 5 mg/ml BSA (Sigma, St. Louis, MO) to a total concentration of 3×10^5 cells/ml. 1.5×10^5 cells were added to the top chamber of the transwell. The T47D cells were allowed to migrate for 20-22 hrs at 37°C. Cells remaining on the top of the polycarbonate membrane were removed. Cells that had migrated to the underside of the membrane were stained with Hema 3 stain set (Fischer Scientific, Pittsburgh, PA). The cells were counted across two diameters, a total of 10 fields, each on duplicate membranes.

Growth in Soft Agar

As previously described (243,244), a bottom layer of 3 mls of Iscove's modified Eagle's medium (Gibco/BRL, Rockville, MD) containing 0.6% agar and 10% fetal bovine serum (Gibco/BRL, Rockville, MD) were plated into 60 mm tissue culture dishes. After the bottom layer had solidified (30 min), 3×10^4 cells were added to 4 mls of Iscove's modified Eagle's medium (Gibco/BRL, Rockville, MD) containing 0.4% Bacto Agar and 5% fetal bovine serum (Gibco/BRL, Rockville, MD). Soft agar assays were maintained in a 37°C/5% CO_2 incubator for 12 days. Cells were fed every two days with 0.5 ml of culture media.

Colonies were stained overnight with 1 mg/ml INT solution (Sigma, St. Louis, IL). Colonies with a diameter of 60 μm or greater were scored.

Proliferation Assay

Cells were trypsinized, counted and replated at a density of 3 x 10^3 cells/well in 96-well dishes. The media of the cells was changed every 2-3 days. At each indicated time point, wells were stained with 5mg/ml MTT (Sigma, St. Louis, IL) at 37° C for 1 hr as previously described (245,246). DMSO was then added to the wells to lyse the cells. Absorbance values at 540 nm over time were compared. This assay was performed in triplicate. For serum independent growth measurements, RPMI1640 containing 0.4% FBS was substituted for the culture media the day after plating. For suspension growth, the cells were plated into 96-well dishes that were coated overnight with 1.2% poly-hydroxyethylmethacrylate (Sigma, St. Louis, MO) in 95% ethanol.

Adhesion Independent Survival

Adhesion independent survival was performed as previously described (21,181). Briefly, 2 x 10^6 cells were suspended for 24 or 48 hrs on tissue culture dishes coated overnight with 4 mls of 1.2% poly-hydroxyethylmethacrylate (Sigma, St. Louis, MO) in 95% ethanol. Apoptotic cells were detected by TUNEL staining using the ApopTag kit (Intergen, Purchase, NY) according to the manufacture's protocol.

In vivo Tumorigenic Assay

Estrogen pellets (0.72 mg; 60-day release pellets) (Innovative Research of America, Sarasota, FL) were implanted by trochar subcutaneously into the scapular space of 5-6 week old female athymic BALB/c mice (Charles River, Wilmington, MA) (247). 10 days after the estrogen pellet implant, cells were injected sc into the flank of the nude mice as described previously (247,248). Prior to injection, 1.2×10^7 cells were suspended in RPMI1640 and mixed 1:1 v/v in Matrigel (BD Biosciences, Bedford, MA). Mice were divided into four groups, each group was injected with T47D/mock cells (5 mice), T47D/FAK cells (5 mice), T47D/SuperFAK cells (8 mice) or T47D/FRNK cells (9 mice). Nude mice were housed at University of North Carolina-Chapel Hill Animal Facility with food and water *ad libitum*. The animals were weighed and tumor size was measured twice a week with vernier calipers. The tumor volumes were calculated according to the formula $V= (4\pi r^3)/3$, where r is half the length of the average of two diameter measurements. The animals were sacrificed 36 days after the tumor cells were injected. Tumors were removed. One half of each tumor was frozen at -80° C and the other half was fixed in formalin (Fischer Scientific, Pittsburgh, PA) for further histopathological analysis. In addition, lungs, lymphatic nodes and livers were also removed and fixed for histopathological analysis.

Results

Expression and biochemical characterization of SuperFAK and FRNK in T47D cells

The T47D breast epithelial cell line used in this study stably expresses the receptor for A type avian retroviruses, Tva. The molecular constructs of interest, FAK, SuperFAK and FRNK, cloned into a replication competent avian retroviral vector, RCAS type A, were successfully expressed in CE cells (3)(data not shown). Seven to 10 days after infection of

T47D/Tva cells with the corresponding retroviruses produced in CE cells, T47D/Tva lysates were Western blotted with a polyclonal FAK antibody. FAK, SuperFAK and FRNK were successfully expressed (Fig. 3-2A). Furthermore, the levels of FAK and SuperFAK expression were equivalent (Fig. 3-2A; left panel).

SuperFAK is an activated mutant of FAK (3). To determine if SuperFAK maintained an elevated catalytic activity when expressed in T47D/Tva breast epithelial cells, *in vitro* kinase assays were performed. The proteins were immunoprecipitated from T47D/Tva lysates expressing empty vector, FAK or SuperFAK using a C-terminal polyclonal FAK antibody, BC4. The immunoprecipitated proteins were incubated in kinase buffer for the indicated times. The kinase reactions were stopped by the addition of Laemmli sample buffer and analyzed by SDS/PAGE and autoradiography (226). Autophosphorylation of endogenous FAK was not very well detected at the exposure shown (Fig. 3-1A), due to the small amount of endogenous FAK that is recovered relative to the exogenously expressed proteins (Fig. 3-1B). However, autophosphorylation of exogenous wild-type FAK was readily detected (Fig. 3-1A). As previously shown in CE cells, a significant increase in autophosphorylation activity was observed in SuperFAK immune complexes compared to wild type FAK (3)(Fig. 3-1A and C). The immune complexes were also Western-blotted with a polyclonal antibody to FAK to demonstrate that equivalent levels of each FAK protein were present in the immunoprecipitates (Fig. 3-1B). Thus, in agreement with the observations in CE cells, SuperFAK has increased *in vitro* kinase activity compared to wild type FAK when expressed in T47D/Tva cells.

FRNK is a naturally occurring dominant negative variant of FAK (5). To ensure that FRNK can also inhibit FAK-mediated signaling in T47D cells, the phosphorylation level of the FAK substrates, paxillin and p130cas, were examined. Paxillin or p130cas were immunoprecipitated from lysates of T47D/Tva expressing FAK, SuperFAK or FRNK. The immune complexes were then Western blotted with a phosphotyrosine antibody. Paxillin phosphorylation was increased upon FAK overexpression (Fig. 3-2B; top panel; lane 2). No further increase in paxillin phosphorylation was observed in SuperFAK expressing T47D/Tva cells (Fig. 3-2B; top panel; lane 3). The level of paxillin phosphorylation was significantly decreased in cells expressing FRNK compared to the mock cells (Fig. 3-2B; top panel; lane 4). The phosphorylation of p130cas was increased upon SuperFAK expression, however FAK and FRNK did not seem to have an affect on the phosphorylation (Fig. 3-2C; top panel). The immune complexes were Western blotted for paxillin or p130cas to verify that equal amounts of protein were recovered (Fig. 3-2 B and C; bottom panels). These observations demonstrate that SuperFAK and FRNK can alter FAK-mediated biochemical signals.

SuperFAK and FRNK alter T47D/Tva cell motility

Since SuperFAK and FRNK could be used to increase and decrease FAK biochemical signaling respectively, their ability to impinge upon FAK-mediated biological processes was tested. FAK is a known mediator of cell motility. The effect of SuperFAK or FRNK expression in T47D/Tva cell motility was investigated using a transwell motility assay as previously described (3). Populations of infected T47D/Tva cells were allowed to migrate for 22 h through a porous transwell membrane coated on the underside with collagen I. The non-

motile cells were removed from the top of the membrane, and cells that migrated to the underside of the membrane were stained and counted. The average fold change in motility was plotted (Fig. 3-3). The motility of T47D/Tva cells was significantly increased by FAK overexpression, which is consistent with previous findings demonstrating a role for FAK in regulating motility in other cell types (6,63,137). Expression of SuperFAK further increased the motility of T47D/Tva cells compared with cells overexpressing wild type FAK, which had been shown previously (3). In contrast, FRNK expression efficiently inhibited T47D/Tva motility consistent with previous findings in other cell types (6). These observations demonstrate the ability of FAK ovexpression, SuperFAK and FRNK to amplify or reduce a FAK-mediated biological response. Furthemore, these results support a role for FAK in regulation of breast cancer epithelial cell motility.

FRNK decreases colony formation of T47D cells in a soft agar system

T47D cells are derived from a mammary breast epithelial tumor (249). These cells exhibit classic cancerous phenotypes which includes adhesion independent growth and tumor formation in nude mice (249,250). The role of FAK signaling in adhesion-independent growth of T47D/Tva cells was investigated by suspending the cells expressing FAK, SuperFAK or FRNK in a soft agar matrix. Twelve days after plating, the colonies that formed were stained, and the number and size of the colonies was scored. There was no difference in the size or number of T47D/Tva colonies formed when either FAK or SuperFAK were expressed compared to the mock infected cells (data not shown). However, the number of colonies was significantly decreased (40%) in FRNK expressing T47D/Tva cells (Fig. 3-4), although no change in the size of the colonies was found (data not shown).

Colonies still present in the FRNK T47D/Tva soft agars were isolated, grown in culture and lysed. Western blot analysis revealed that the remaining colonies had decreased or no FRNK expression compared to the parental FRNK expressing T47D/Tva cells (data not shown). Ultimately, these observations suggest an important role for FAK signaling in adhesion independent growth.

In order to investigate the mechanism through which FRNK may be inhibiting adhesion independent growth, we took advantage of a series of FRNK mutants which were previously characterized (251). FRNK HK (H1026/K1033), ML (L1028/L1035), and TRP (E949/K956/R963), contain several point mutations in the FAT sequence that affects paxillin binding and/or focal adhesion localization. Although HK and TRP subcellular localization is unaffected, ML has lost its ability to localize to focal adhesions (251). These defects were correlated with a reduced capacity to act as a dominant negative. FRNK HK, ML and TRP were successfully expressed in T47D/Tva cells (Fig. 3-5). The ability of each one of the FRNK mutants to inhibit the adhesion independent growth of the T47D/Tva cells was examined. The expression of FRNK HK had no effect on the colony formation ability of the T47D/Tva cells, suggesting that the inhibitory effect of FRNK may be due to its dominant negative activity. However, ML and TRP expression decreased the number of colonies formed to comparable FRNK inhibition levels (Fig. 3-5). Interestingly, the results obtained from the FRNK mutant studies suggest that neither focal adhesion targeting nor paxillin binding are critical determinants for adhesion independent growth.

FRNK has no effect on the proliferation or survival of T47D/Tva cells

FAK signaling has been implicated in the regulation of cell cycle progression (23,239). In addition, FAK is a known positive signaling component of adhesion-mediated survival (21). Furthermore, FRNK has been showed to cause cells to undergo apoptosis (180). The inability of FRNK expressing T47D/Tva cells to form colonies in soft agar could be explained by a dominant negative effect of FRNK on FAK-mediated proliferation or survival. Therefore, the effect of FRNK expression on growth and survival of T47D/Tva cells was investigated, to determine the mechanism of inhibition of adhesion independent growth.

The growth of FRNK expressing T47D/Tva cells were cultured in multi-well dishes in the presence of complete media (10% FBS) was first assessed (Fig. 3-6 A). One distinctive characteristic of tumor cells and T47D cells is their ability to proliferate even in an environment with minimal amounts of growth factors. Thus, the proliferative activity of T47D/Tva cells expressing FRNK in the presence of low serum (0.4% FBS) was also measured (Fig. 3-6 C). The proliferation of FRNK expressing T47D cells grown "in suspension" on poly-HEMA coated multi-well dishes was also investigated. T47D/Tva cells expressing empty vector or FRNK were plated in poly-HEMA coated dishes, onto which cells are unable to adhere. Suspended cells were grown in the presence of high (10% FBS) or low (0.4% FBS) levels of serum (Fig. 3-6 B and data not shown). The number of cells present in a well was quantified using a cell viability stain at the indicated days after plating. The quantification readings were plotted to visualize and compare the growth curves of T47D/Tva expressors (Fig. 3-6 and data not shown). No significant difference in the slope of

the growth curves was observed, suggesting that FRNK expression has no effect on the proliferation of T47D/Tva cell in standard growth conditions, in low serum or in suspension. Therefore reduced colony formation could not be explained by a decreased rate of proliferation.

A second possible mechanism of FRNK action may be on the survival of T47D cells. FRNK has been shown to inhibit survival signals and cause apoptosis in certain circumstances (180). If FRNK were causing high rates of apoptosis in our system, a selection against FRNK expressing cells would be expected. However, the expression of FRNK in cultured T47D/Tva cells remains stable and constant as long as 9 weeks after infection (Fig. 3-7 B). This observation suggests that FRNK is not affecting the survival of T47D/Tva cells in culture. However, since the FRNK effects were observed in an adhesion independent growth assay, FRNK may impinge differentially on the survival of cells that were grown in the absence of an adhesion signal. Cells were held in suspension for 24 and 48 hrs, at which time cells were collected and apoptotic cell numbers were measured by TUNEL staining. No increase in apoptosis was observed in FRNK expressing cells compared to control cells (Fig. 3-5 A). The murine breast cells, HC11, were used as a control for suspension-mediated cell death. DNase treated T47D cells were used as a positive TUNEL staining control. Thus, in accordance with its sustained expression in cultured cells, and the growth in suspension measurements, FRNK has no effect on the survival of T47D cells grown in an adhesion-independent manner. From these observations we can conclude that FRNK is not impinging on the survival or growth rates of T47D cells grown in the absence of adhesion signals.

SuperFAK expression increases the tumorigenicity of T47D/Tva cells in vivo

The most distinct characteristic of transformed or tumorigenic cells is their ability to form tumors in nude mice. Although estrogen-dependent, T47D cells can form palpable tumors in mice. In order to determine the role of FAK signaling in tumorigenicity and progression, T47D/Tva cells expressing FAK, SuperFAK or FRNK were subcutaneously injected in athymic mice. The appearance and growth rate of the tumors were measured bi-weekly for about 6 weeks (Fig. 3-8). The tumor growth rates and the final tumor sizes were compared. Neither FAK nor FRNK had a significant effect on the size of the tumors compared to vector expressing cells, SuperFAK tumors consistently showed an increase in tumor volume. This observation suggests that the increase in FAK signaling attained by expressing SuperFAK in T47D/Tva cells was sufficient to increase the tumorigenicity of T47D/Tva cells.

Discussion

In this study, we investigated the role of FAK signaling in cancer progression by exploiting a cell model system. Wild type FAK, SuperFAK and FRNK were used to manipulate FAK signaling in the breast cancer epithelial cell line, T47D. In accordance with previous investigations, FAK, SuperFAK and FRNK were shown to have robust effects on T47D/Tva cell motility (3,6). Therefore, SuperFAK and FRNK were used as molecular tools to manipulate FAK biochemical and biological signaling in this study. The adhesion independent growth of cancer cells was partially inhibited by FRNK. Intriguingly, FRNK expression had no effect on proliferation or survival of T47D cells either in culture or in cells grown in the absence of adhesion. SuperFAK in turn was able to elevate the tumor growth of

T47D cells *in vivo*. The observations of this study suggest a role for FAK signaling in the acquisition of cancer phenotypes during tumor cell progression.

The attenuation of FAK signaling by antisense techniques has previously been shown to inhibit adhesion-independent growth of non-small cell lung cancer cells (186). FAK may contribute to adhesion-independent growth of cancer cells by promoting cellular processes it regulates in normal cells, including cell cycle progression, and survival (6,21,23,239). Furthermore, FRNK can induce apoptosis in breast cancer cells, suggesting a role for endogenous FAK in promoting cell survival (6,20,97,180,181). Unexpectedly, FRNK expression had no effect on the rate of proliferation or the survival of the T47D/Tva breast cancer epithelial cells in cultured conditions or in cells grown in the absence of an adhesion signal. There could be several reasons for the conflicting FRNK effects on cell proliferation and survival. One possibility is the existence of cell type specific responses. Furthermore, it must be noted that, as mentioned above, the amount of FRNK expression in the T47D cells was unable to completely block substrate phosphorylation. In contrast, the earlier studies in which FRNK inhibitory effects on proliferation and survival were observed took advantage of highly efficient protein delivery systems (6,180). Therefore, the results could be due to efficiency of inhibition. Most interestingly, these observations suggest that an alternative mechanism of action might exist for FRNK to mediate its inhibitory effects in soft agar growth.

In agreement with this observation (186), inhibition of FAK signaling by FRNK leads to a reduced ability of breast cancer epithelial cells to form colonies in soft agar. However, it must be noted that FRNK does not completely abolish colony formation of T47D cells. There are several possible explanations for the incomplete block of growth in soft agar. First, we

showed that several persisting colonies had decreased or loss of FRNK expression. This observation suggests that only the colonies that did not express any or sufficient levels of FAK were able to form colonies. Thus, The FRNK expressing cells were inhibited in their ability to grow in soft agar. Second, FRNK also did not completely abolish substrate phosphorylation offering an explanation for partial inhibition of growth in soft agar by FRNK. It may be possible to further inhibit the adhesion-independent growth of the cells by completely inhibiting substrate phosphorylation with increased FRNK expression, or utilizing antisense or RNAi techniques as an alternative way to abolish FAK signaling. In addition, the failure of FRNK to impinge upon cell proliferation and/or survival may in turn illustrate the reason for the modest inhibition of T47D colony formation. Alternatively, the inability of FRNK to entirely block colony formation may suggest the involvement of additional redundant or parallel signals that in combination with FAK signaling might contribute to the adhesion-independent growth phenotype of the breast cancer cells.

Overall, the results of the FRNK mutants on adhesion independent growth suggest that neither paxillin binding nor focal adehsion localization are not critical determinants of FRNK inhibition. The inability of the FRNK HK mutant to negatively impinge upon soft colony growth of the breast cancer epithelial cells suggests interesting mechanisms of action. Although HK, like wild type FRNK, is able to localize to focal adhesions it shows decreased ability for paxillin binding (251). Furthermore, FRNK HK does not have a strong inhibitory effect on substrate phosphorylation (251). These observations suggest that focal adhesion localization, paxillin binding and most importantly the dominant activity of FRNK *per se* could be responsible for the effect on adhesion independent growth. However, the successful inhibition of T47D adhesion independent growth by ML, suggests that focal adhesion

targeting might not be necessary for the inhibitory effect. In addition, TRP is also able to inhibit colony formation. Both ML and TRP have reduced paxillin binding and decreased dominant negative activity suggesting that neither of these abilities are the sole mediators of the adhesion independent growth inhibition by FRNK. Thus, an additional mechanism of action or multiple mechanisms might be responsible for the FRNK effects. It may be interesting to determine the p130cas and talin binding activities of the FRNK mutants to determine if the ability of FRNK and the FRNK mutants to bind and sequester other proteins may be linked to their effects on adhesion independent growth of T47D cells.

The animal studies demonstrate that increased FAK signaling by SuperFAK expression increases the growth of the breast cancer epithelial tumors. These results are in accordance with previous observations in which FAK overexpression alone was sufficient to increase astrocytoma tumor growth in mice (174). FAK overexpression potentially contributes to tumorigenesis *in vivo* by promoting in the animal cellular processes it regulates in normal cells, including cell proliferation and/or survival. MDCK cells expressing the activated FAK mutant, CD2FAK, form tumors in nude mice and this effect could be a result of the inhibition of anoikis, which in turn allows for adhesion-independent growth (21). The increased astrocytoma growth upon FAK overexpression has been associated with increased tumor cell proliferation (174). Furthermore, FRNK-mediated inhibition of carcinoma cell tumors has been associated with an inhibition of cell proliferation (199). The lack of FAK or FRNK effect in the animal studies may also suggest in this system FAK alone is not sufficient to impinge upon tumorigenesis or simply that the neither FAK nor FRNK expression sufficiently regulates FAK signaling enough to have an affect on tumor growth, which correlates with some of the biochemical effects. Our studies demonstrate that

SuperFAK expression has no effect on the growth or survival of the breast cancer epithelial cells in cultured conditions or in the absence of adhesion, regardless of the presence or absence of serum (data not shown). Therefore, our observations suggest cell specific responses. Furthermore, it cannot always be presumed that cellular processes such as proliferation and survival are being regulated in the same way in culture as in the mice. But most importantly, since increased tumor growth by SuperFAK does not correlate with changes in cellular proliferation nor survival, these observations suggest that FAK signaling might be impinging on other cellular or physiological processes, thus implicating additional mechanisms of action of FAK in tumorigenesis. Interestingly, FRNK but not SuperFAK had an effect on adhesion-independent growth of the breast epithelial cells but only SuperFAK affected tumor growth. These observations suggest that the effect of increased FAK signaling upon SuperFAK expression may be in effect using a different mechanism other than proliferation, survival, or adhesion independent growth to impinge on tumor growth in mice.

Finally, cooperation between multiple signaling pathways may contribute to the deregulation of cellular processes associtate with cellular transformation and cancer. There is growing evidence to suggest that FAK mediates crosstalk between the integrin and growth factor pathways (58,189,207). Inappropriate activation of growth factor receptor pathways has been linked to a variety of human tumors (2). Interestingly, FAK has been found to associate with and be activated by growth factor receptors (2). An activated mutant of FAK was unable to affect adhesion independent growth properties without the introduction of a second signal, Ras, which is an important downstream effector of growth factor receptors (185). Furthermore, FAK signaling has been implicated in growth factor-mediated regulation of normal and cancerous cellular processes including motility, invasion and survival

(58,189,193). The inability of FRNK to completely inhibited soft agar colony formation of T47D/Tva cells may thus be in part explained by the existence of additional cooperating signals, that with FAK, act on the adhesion independent growth of tumor cells. Furthermore, the addition of a second signal in conjunction with SuperFAK expression may have had a more robust effect on the adhesion independent growth and tumor growth of SuperFAK expressing T47D/Tva cells. Thus, the cooperation of FAK and growth factors may a mechanism for tumor formation and progression. Furthermore, although FAK may not act as a true oncogene (198), this study demonstrates that FAK may contribute to or be permissive for tumor progression, suggesting that FAK may be a reasonable target for therapeutic intervention.

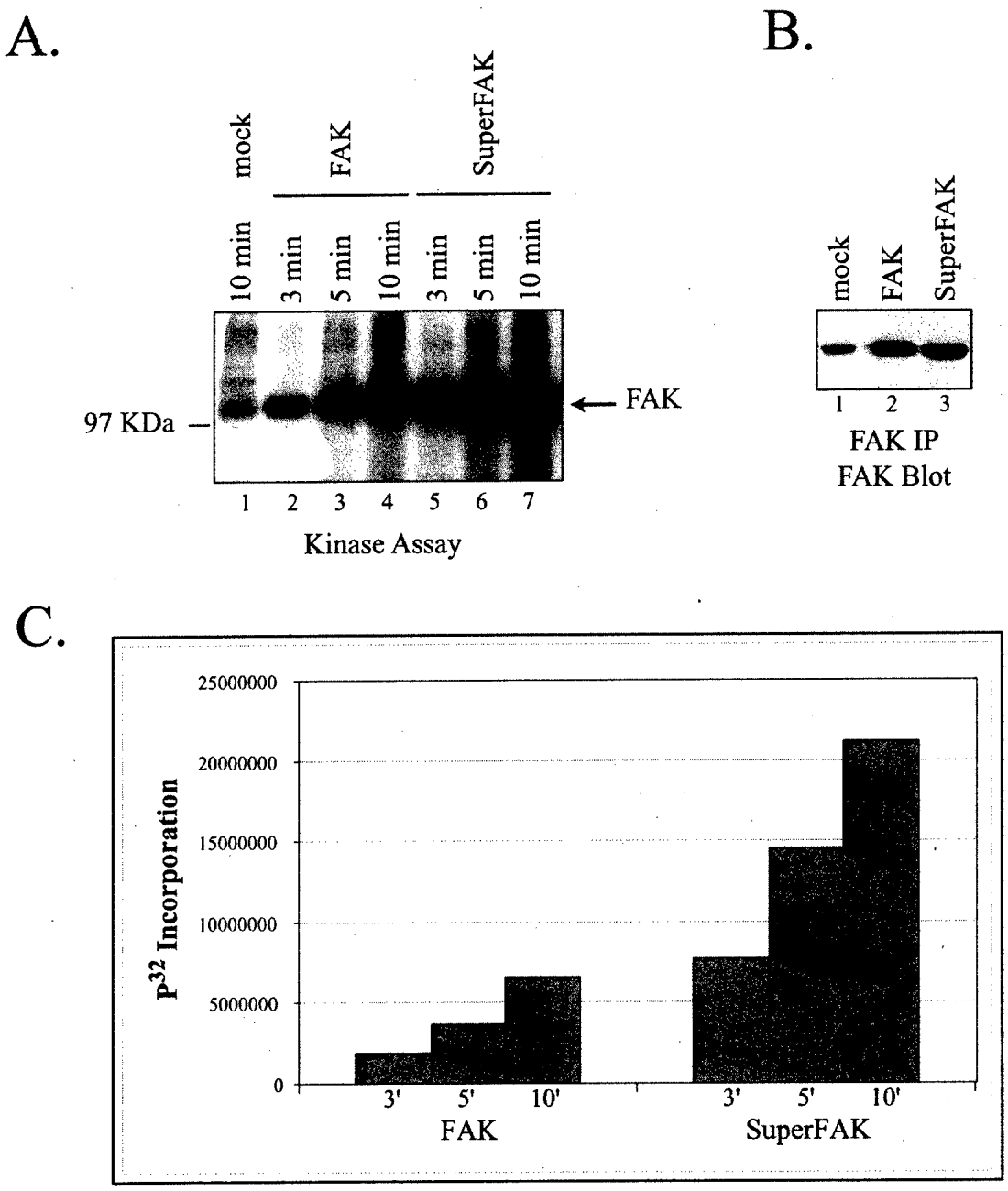


Figure 3-1. In Vitro Kinase Activity. A, The in vitro autophosphorylation activity of wild type FAK and SuperFAK was determined using an immune complex kinase assay. FAK was immunoprecipitated from T47D/Tva cell lysates (0.5-1 mg) expressing vector alone (lane 1), FAK (lane 2-4), SuperFAK (lane 5-7), using a polyclonal FAK antibody, BC4. The immune complexes were incubated in kinase buffer containing ^{32}P-γATP for 3, 5 or 10 minutes. The samples were subjected to SDS-PAGE and visualized by autoradiography. The position of FAK is indicated by an arrow on the right. The 97 KDa molecular weight marker is indicated on the left. **B**, The immune complexes were also Western blotted using a polyclonal FAK antibody, BC4, to ensure equal loading. **C**, ^{32}P incorporation was quantified by phosphorimager analysis.

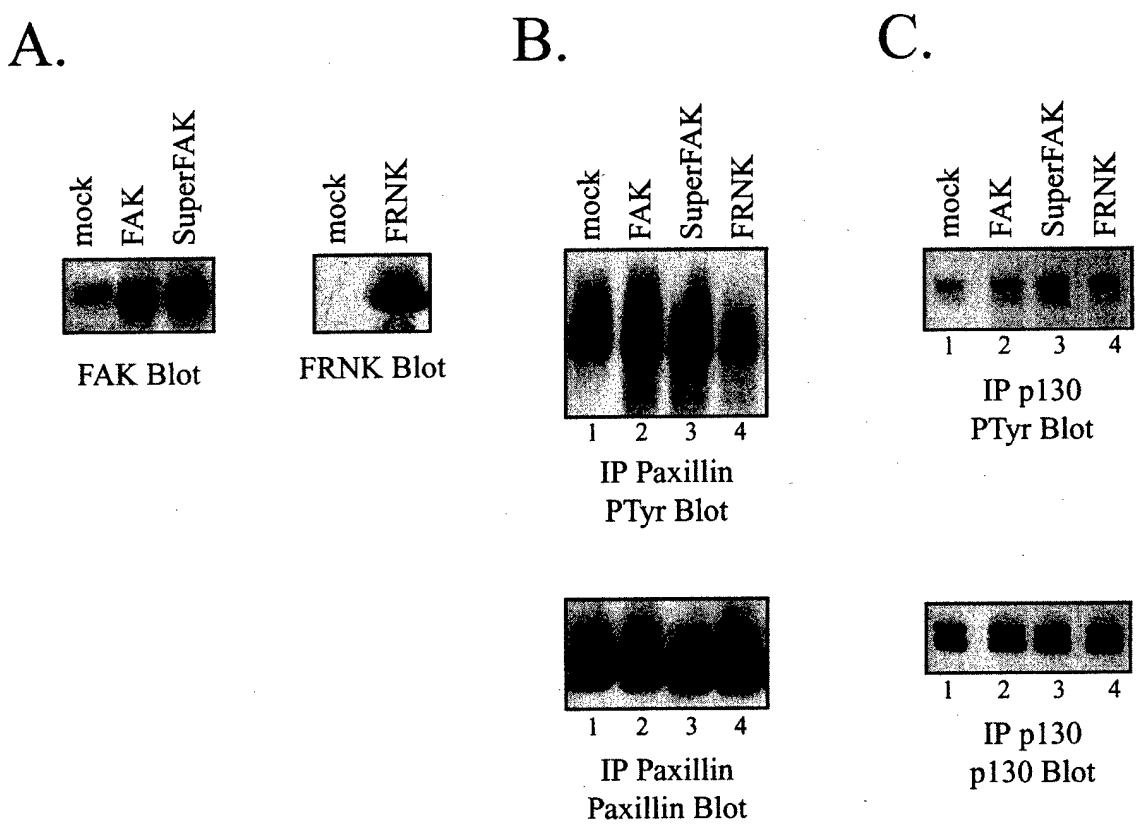


Figure 3-2. Expression of FAK, SuperFAK and FRNK in T47D/Tva cells and its effect on substrate phosphorylation. A, Expression of FAK, SuperFAK (left panel) and FRNK (right panel) was detected by Western blot analysis. Seven days after infection, lysates (25 μg) of T47D/Tva cells were Western blotted with a FAK polyclonal antibody, BC4. **B-C**, Paxillin (**B**) and p130cas (**C**) were immunoprecipitated from T47D/Tva cells (0.5-1 mg) expressing vector alone (lane 1), FAK (lane 2), SuperFAK (lane 3) or FRNK (lane 4). The immune complexes were Western blotted with a phosphotyrosine antibody, RC20 (B-C; top panels). The nitrocellulose membranes were stripped and reprobed for paxillin and p130cas to ensure equal amounts of protein were being analyzed (**B-C**; bottom panels).

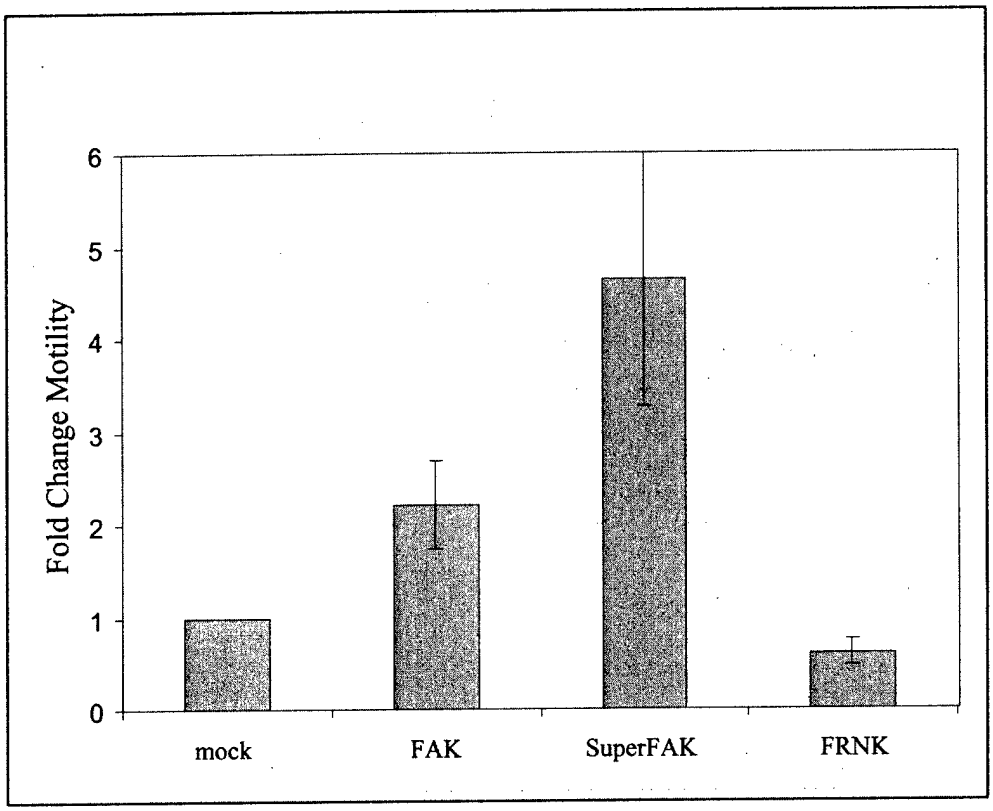
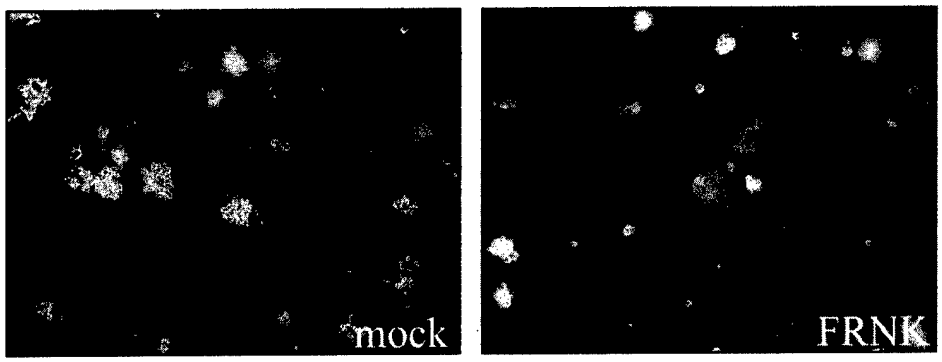


Figure 3-3. Effects of FAK, SuperFAK and FRNK expression on T47D/Tva cell motility. The motility of T47D/Tva cells expressing empty vector (mock), FAK, SuperFAK or FRNK was measured in a transwell system. Cells were allowed to migrate to the underside of a collagen coated transwell membrane for 20-22 hrs. The number of cells that reached the underside of the membrane were counted. The average fold change in migration from 9 experiments is shown – standard error.

A.



B.

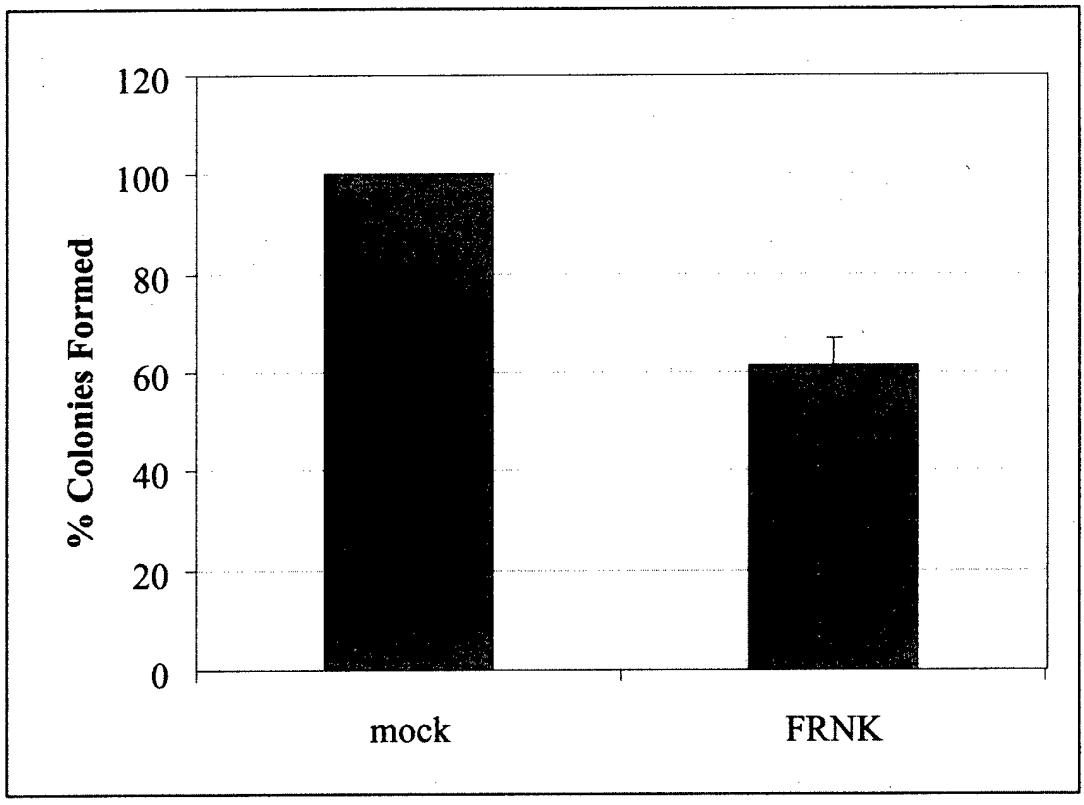
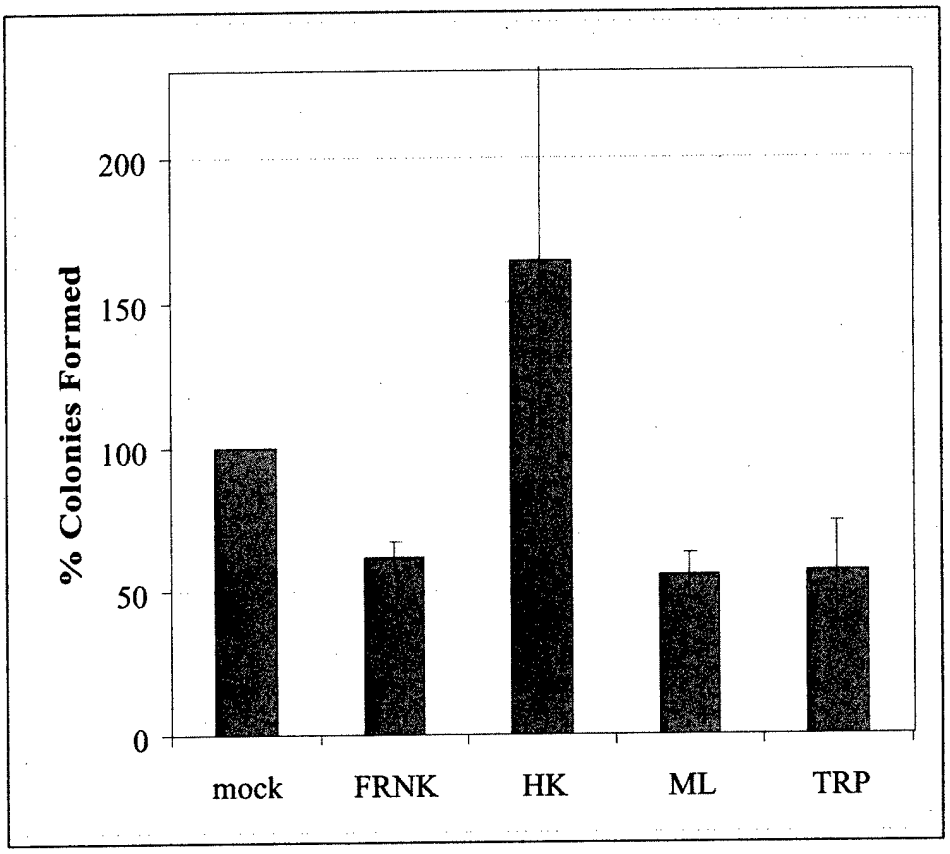
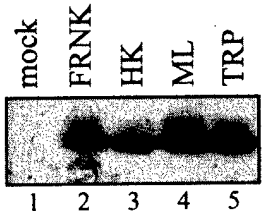


Figure 3-4. Decreased adhesion independent growth of FRNK expressing T47D/Tva cells. T47D/Tva cells ($3x10^4$) expressing wild type FAK or FRNK were suspended in a soft agar matrix (0.4% Bacto Agar) for 12 days. The colonies formed were photographed (**A**) and counted. **B**, The results are expressed as the mean percentage of number of colonies formed, relative to the parental T47D/Tva cells (100%) ± standard error (n=6).

A.



B.



FRNK Blot

Figure 3-5. Effect of FRNK mutants on the adhesion independent growth of T47D/Tva cells. A, T47D/Tva cells ($3x10^4$) expressing wild type FAK, FRNK, $FRNK^{H1026/K1033}$ (HK), $FRNK^{L1028/L1035}$ (ML), or $FRNK^{E949/K956/R963}$ (TRP) were suspended in a soft agar matrix (0.4% Bacto Agar) for 12 days. The colonies formed were scored. The results are expressed as the mean percentage of number of colonies formed relative to the parental T47D/Tva cells (100%) ± standard error. **B,** T47D/Tva lysates (25 µg) of cells expressing vector alone (mock) (lane 1), FRNK (lane 2), $FRNK^{H1026/K1033}$ (HK) (lane 3), $FRNK^{L1028/L1035}$ (ML) (lane 4), or $FRNK^{E949/K956/R963}$ (TRP) (lane 5) were Western blotted with a FAK polyclonal antibody, BC4, to ensure equivalent amounts of the proteins were being expressed.

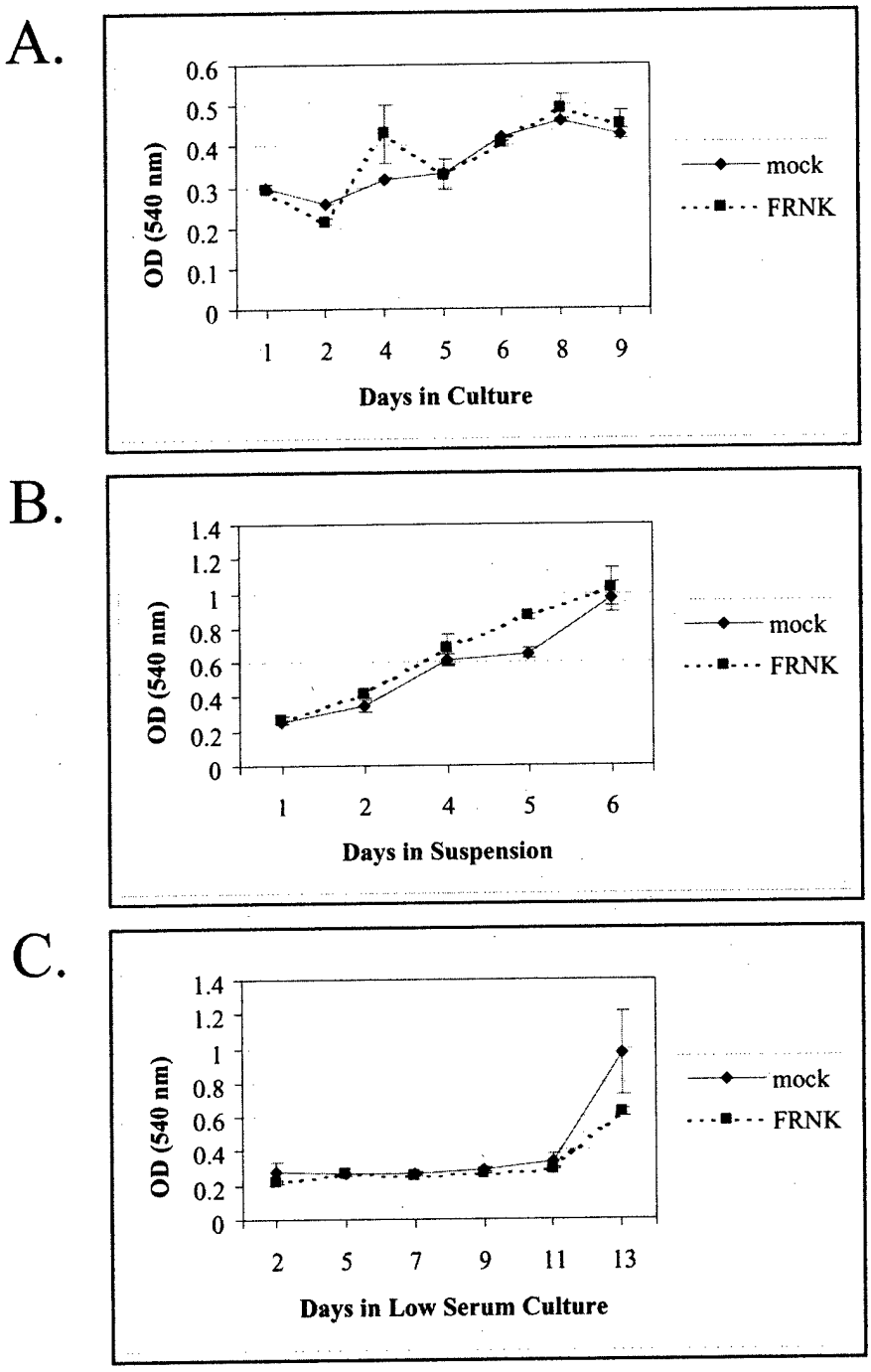
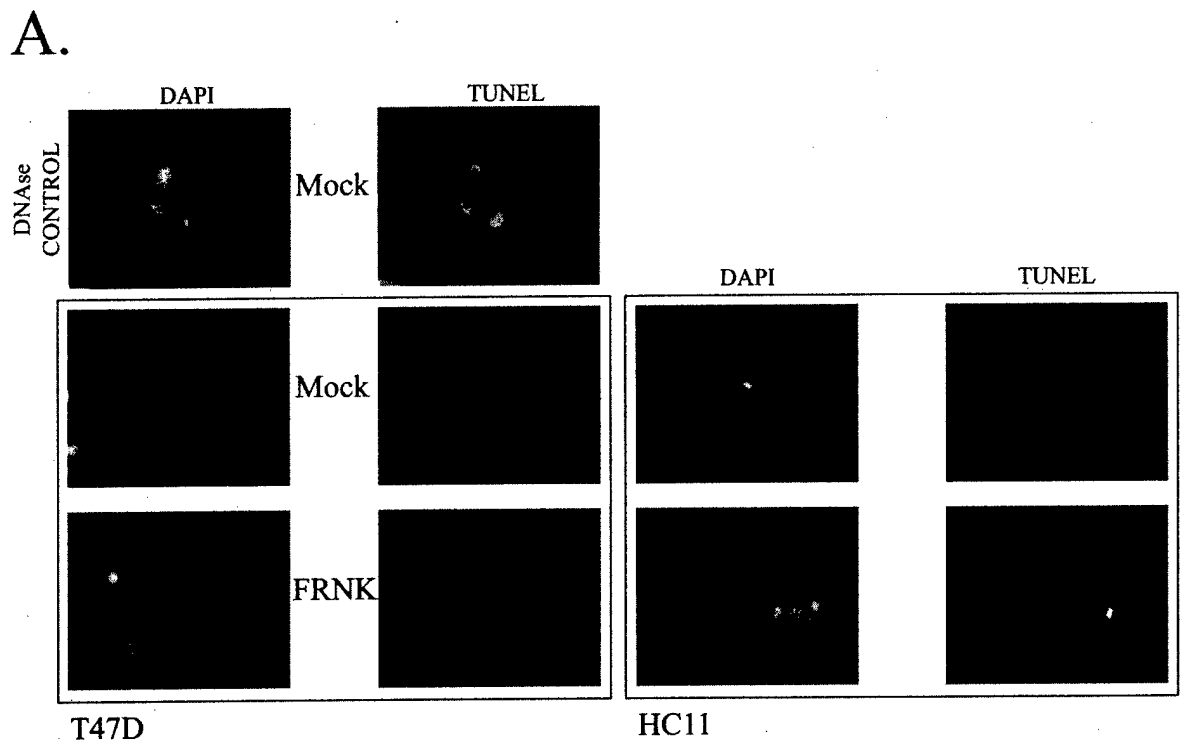


Figure 3-6. FRNK expression does not affect the proliferation of T47D/Tva cells. T47D/Tva cells ($3x10^3$ cells/well) expressing vector alone (mock) (continous lines) or FRNK (dashed lines) were plated in multi-well plates. Cells were either grown in culture media (10% FBS) (**A**), in suspension in complete media (**B**) or cultured in low serum media (0.4% FBS) (**C**). At the indicated times after plating, cells were stained with MTT and absorbance at 540 nm measured. A representative experiment is shown. The results are expressed as the mean of triplicate wells – standard error.



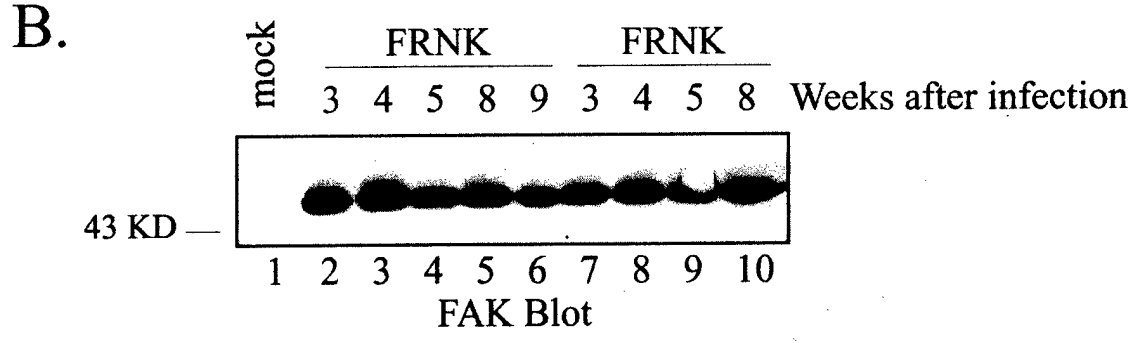
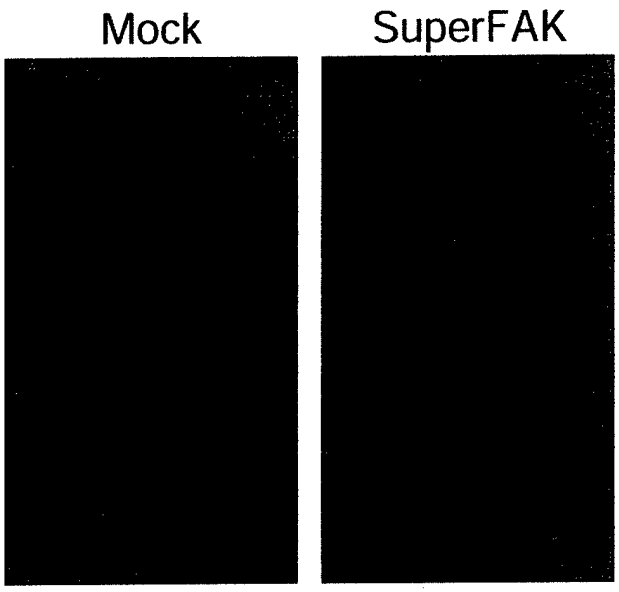


Figure 3-7. FRNK expression has no effect on the adhesion independent survival of T47D/Tva cells. A, T47D/Tva cells expressing vector alone (mock) or FRNK were grown in suspension for 24 or 48 hrs. Cells were collected and apoptotic numbers were determined by TUNEL staining. DAPI staining was used to identify cell nuclei. As a positive control for TUNEL staining, T47D/Tva cells were pre-treated with DNAse. As a control for adhesion mediated apoptosis, HC11 cells were used. A representative experiment is shown. **B,** T47D/Tva cells expressing vector alone (mock) (lane 1) or FRNK (lanes 2-10) were lysed at the indicated time after infection. Lysates from two independent were analyzed. Lysates (25 μg) were Western blotted with a FAK polyclonal antibody, BC4, to determine if growth selection against FRNK expressing cells was occurring.

A.



B.

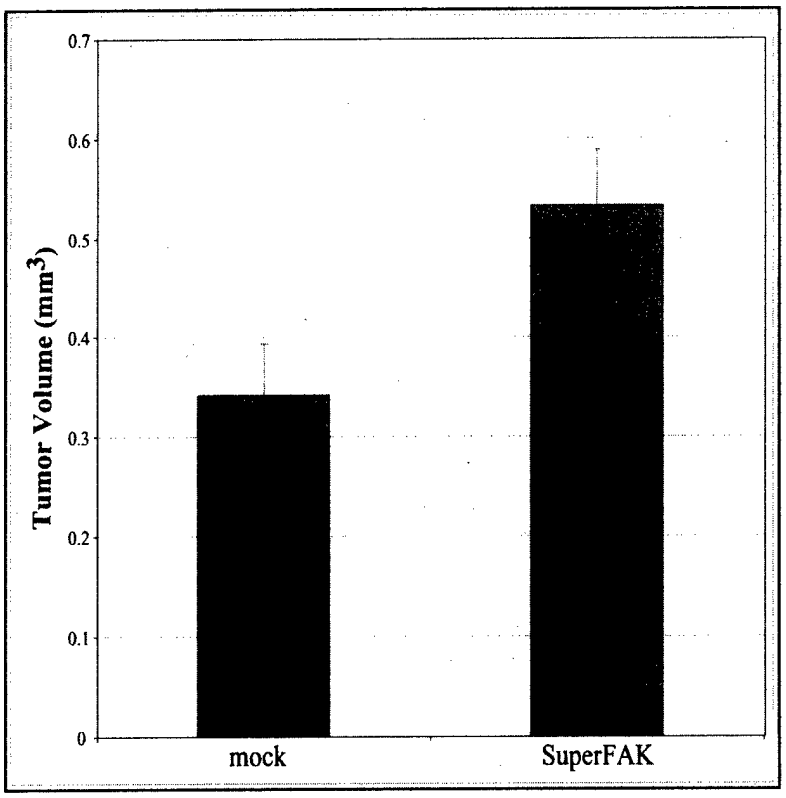


Figure 3-8. SuperFAK increases T47D/Tva tumor growth. T47D/Tva cells ($1.2x10^7$ cells) expressing vector alone (mock) or SuperFAK were injected subcutaneously into the flank of 8 week old athymic female BALB/c mice. Tumor volume was measured. **A**, Representative animals at 36 days post-injection are shown. **B**, The results are expressed as average tumor volumes of 5 mice (10 tumors) – standard error.

REFERENCES

1. Schaller, M. D. (2001) *Biochim.Biophys.Acta* **1540,** 1-21

2. Gabarra-Niecko, V., Schaller, M. D., and Dunty, J. M. (2003) *Cancer Metastasis Rev.* **22,** 359-374 (In Press)

3. Gabarra-Niecko, V., Keely, P. J., and Schaller, M. D. (2002) *Biochem.J.* **365,** 591-603

4. Schaller, M. D., Borgman, C. A., and Parsons, J. T. (1993) *Mol.Cell Biol.* **13,** 785-791

5. Richardson, A. and Parsons, T. (1996) *Nature* **380,** 538-540

6. Gilmore, A. P. and Romer, L. H. (1996) *Mol.Biol.Cell* **7,** 1209-1224

7. Kanner, S. B., Reynolds, A. B., Vines, R. R., and Parsons, J. T. (1990) *Proc.Natl.Acad.Sci.U.S.A* **87,** 3328-3332

8. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T. (1992) *Proc.Natl.Acad.Sci.U.S.A* **89,** 5192-5196

9. Maher, P. A., Pasquale, E. B., Wang, J. Y., and Singer, S. J. (1985) *Proc.Natl.Acad.Sci.U.S.A* **82,** 6576-6580

10. Hynes, R. O. (1992) *Cell* **69,** 11-25

11. Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988) *Annu.Rev.Cell Biol.* **4,** 487-525

12. Guan, J. L., Trevithick, J. E., and Hynes, R. O. (1991) *Cell Regul.* **2,** 951-964

13. Kornberg, L. J., Earp, H. S., Turner, C. E., Prockop, C., and Juliano, R. L. (1991) *Proc.Natl.Acad.Sci.U.S.A* **88,** 8392-8396

14. Golden, A., Brugge, J. S., and Shattil, S. J. (1990) *J.Cell Biol.* **111,** 3117-3127

15. Burridge, K., Turner, C. E., and Romer, L. H. (1992) *J.Cell Biol.* **119,** 893-903

16. Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992) *J.Biol.Chem.* **267,** 23439-23442

17. Guan, J. L. and Shalloway, D. (1992) *Nature* **358,** 690-692

18. Hanks, S. K., Calalb, M. B., Harper, M. C., and Patel, S. K. (1992) *Proc.Natl.Acad.Sci.U.S.A* **89,** 8487-8491

19. Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992) *J.Cell Biol.* **119,** 905-912

20. Hungerford, J. E., Compton, M. T., Matter, M. L., Hoffstrom, B. G., and Otey, C. A. (1996) *J.Cell Biol.* **135,** 1383-1390

21. Frisch, S. M., Vuori, K., Ruoslahti, E., and Chan-Hui, P. Y. (1996) *J.Cell Biol.* **134,** 793-799

22. Romer, L. H., McLean, N., Turner, C. E., and Burridge, K. (1994) *Mol.Biol.Cell* **5,** 349-361

23. Zhao, J. H., Reiske, H., and Guan, J. L. (1998) *J.Cell Biol.* **143,** 1997-2008

24. Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R., and Parsons, J. T. (1994) *Mol.Cell Biol.* **14,** 1680-1688

25. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der, G. P. (1994) *Nature* **372,** 786-791

26. Calalb, M. B., Polte, T. R., and Hanks, S. K. (1995) *Mol.Cell Biol.* **15,** 954-963

27. Calalb, M. B., Zhang, X., Polte, T. R., and Hanks, S. K. (1996) *Biochem.Biophys.Res.Commun.* **228,** 662-668

28. Pawson, T. and Gish, G. D. (1992) *Cell* **71,** 359-362

29. Cobb, B. S., Schaller, M. D., Leu, T. H., and Parsons, J. T. (1994) *Mol.Cell Biol.* **14,** 147-155

30. Schlaepfer, D. D. and Hunter, T. (1996) *Mol.Cell Biol.* **16,** 5623-5633

31. Chen, H. C. and Guan, J. L. (1994) *Proc.Natl.Acad.Sci.U.S.A* **91,** 10148-10152

32. Thomas, J. W., Ellis, B., Boerner, R. J., Knight, W. B., White, G. C., and Schaller, M. D. (1998) *J.Biol.Chem.* **273,** 577-583

33. Harte, M. T., Hildebrand, J. D., Burnham, M. R., Bouton, A. H., and Parsons, J. T. (1996) *J.Biol.Chem.* **271,** 13649-13655

34. Polte, T. R. and Hanks, S. K. (1995) *Proc.Natl.Acad.Sci.U.S.A* **92,** 10678-10682

35. Cary, L. A., Han, D. C., Polte, T. R., Hanks, S. K., and Guan, J. L. (1998) *J.Cell Biol.* **140,** 211-221

36. Hildebrand, J. D., Schaller, M. D., and Parsons, J. T. (1995) *Mol.Biol.Cell* **6,** 637-647

37. Hildebrand, J. D., Schaller, M. D., and Parsons, J. T. (1993) *J.Cell Biol.* **123,** 993-1005

38. Chen, H. C., Appeddu, P. A., Parsons, J. T., Hildebrand, J. D., Schaller, M. D., and Guan, J. L. (1995) *J.Biol.Chem.* **270,** 16995-16999

39. Sasaki, H., Nagura, K., Ishino, M., Tobioka, H., Kotani, K., and Sasaki, T. (1995) *J.Biol.Chem.* **270,** 21206-21219

40. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995) *Nature* **376,** 737-745

41. Yu, H., Li, X., Marchetto, G. S., Dy, R., Hunter, D., Calvo, B., Dawson, T. L., Wilm, M., Anderegg, R. J., Graves, L. M., and Earp, H. S. (1996) *J.Biol.Chem.* **271,** 29993-29998

42. Avraham, S., London, R., Fu, Y., Ota, S., Hiregowdara, D., Li, J., Jiang, S., Pasztor, L. M., White, R. A., and Groopman, J. E. (1995) *J.Biol.Chem.* **270,** 27742-27751

43. Hayashi, I., Vuori, K., and Liddington, R. C. (2002) *Nat.Struct.Biol.* **9,** 101-106

44. Arold, S. T., Hoellerer, M. K., and Noble, M. E. (2002) *Structure.(Camb.)* **10,** 319-327

45. Liu, G., Guibao, C. D., and Zheng, J. (2002) *Mol.Cell Biol.* **22,** 2751-2760

46. Nowakowski, J., Cronin, C. N., McRee, D. E., Knuth, M. W., Nelson, C. G., Pavletich, N. P., Rogers, J., Sang, B. C., Scheibe, D. N., Swanson, R. V., and Thompson, D. A. (2002) *Structure.(Camb.)* **10,** 1659-1667

47. Girault, J. A., Labesse, G., Mornon, J. P., and Callebaut, I. (1999) *Trends Biochem.Sci.* **24,** 54-57

48. Chishti, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N., Chasis, J. A., Conboy, J. G., Gascard, P., Takakuwa, Y., Huang, S. C., Benz, E. J., Jr., Bretscher, A., Fehon, R. G., Gusella, J. F., Ramesh, V., Solomon, F., Marchesi, V. T., Tsukita, S., Tsukita, S., Hoover, K. B., and . (1998) *Trends Biochem.Sci.* **23,** 281-282

49. Hamada, K., Shimizu, T., Matsui, T., Tsukita, S., and Hakoshima, T. (2000) *EMBO J* **19,** 4449-4462

50. Pearson, M. A., Reczek, D., Bretscher, A., and Karplus, P. A. (2000) *Cell* **101,** 259-270

51. Hasson, T. (1999) *Curr.Biol.* **9,** R838-R841

52. Gusella, J. F., Ramesh, V., MacCollin, M., and Jacoby, L. B. (1999) *Biochim.Biophys.Acta* **1423,** M29-M36

53. Ward, A. C., Touw, I., and Yoshimura, A. (2000) *Blood* **95,** 19-29

54. Zhou, Y. J., Chen, M., Cusack, N. A., Kimmel, L. H., Magnuson, K. S., Boyd, J. G., Lin, W., Roberts, J. L., Lengi, A., Buckley, R. H., Geahlen, R. L., Candotti, F., Gadina, M., Changelian, P. S., and O'Shea, J. J. (2001) *Mol.Cell* **8,** 959-969

55. Louvet-Vallee, S. (2000) *Biol.Cell* **92,** 305-316

56. Schaller, M. D., Otey, C. A., Hildebrand, J. D., and Parsons, J. T. (1995) *J.Cell Biol.* **130,** 1181-1187

57. Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H., and Schlaepfer, D. D. (2000) *Nat.Cell Biol.* **2,** 249-256

58. Golubovskaya, V., Beviglia, L., Xu, L. H., Earp, H. S., III, Craven, R., and Cance, W. (2002) *J.Biol.Chem.* **277,** 38978-38987

59. Chen, R., Kim, O., Li, M., Xiong, X., Guan, J. L., Kung, H. J., Chen, H., Shimizu, Y., and Qiu, Y. (2001) *Nat.Cell Biol.* **3,** 439-444

60. Dunty, J. M., Gabarra-Niecko, V., and Schaller, M. D. (2003) *Mol.Cell Biol.* Submitted

61. Fiedorek, F. T., Jr. and Kay, E. S. (1995) *Mamm.Genome* **6,** 123-126

62. Agochiya, M., Brunton, V. G., Owens, D. W., Parkinson, E. K., Paraskeva, C., Keith, W. N., and Frame, M. C. (1999) *Oncogene* **18,** 5646-5653

63. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) *Nature* **377,** 539-544

64. Burgaya, F. and Girault, J. A. (1996) *Brain Res.Mol.Brain Res.* **37,** 63-73

65. Derkinderen, P., Toutant, M., Burgaya, F., Le Bert, M., Siciliano, J. C., de, F., V, Gelman, M., and Girault, J. A. (1996) *Science* **273,** 1719-1722

66. Burgaya, F., Toutant, M., Studler, J. M., Costa, A., Le Bert, M., Gelman, M., and Girault, J. A. (1997) *J.Biol.Chem.* **272,** 28720-28725

67. Toutant, M., Studler, J. M., Burgaya, F., Costa, A., Ezan, P., Gelman, M., and Girault, J. A. (2000) *Biochem.J.* **348 Pt 1,** 119-128

68. Nolan, K., Lacoste, J., and Parsons, J. T. (1999) *Mol.Cell Biol.* **19,** 6120-6129

69. Richardson, A., Malik, R. K., Hildebrand, J. D., and Parsons, J. T. (1997) *Mol.Cell Biol.* **17,** 6906-6914

70. Shen, Y. and Schaller, M. D. (1999) *Mol.Biol.Cell* **10,** 2507-2518

71. Zachary, I., Sinnett-Smith, J., and Rozengurt, E. (1992) *J.Biol.Chem.* **267,** 19031-19034

72. Kumagai, N., Morii, N., Fujisawa, K., Yoshimasa, T., Nakao, K., and Narumiya, S. (1993) *FEBS Lett.* **329,** 273-276

73. Seufferlein, T. and Rozengurt, E. (1994) *J.Biol.Chem.* **269,** 9345-9351

74. Polte, T. R., Naftilan, A. J., and Hanks, S. K. (1994) *J.Cell Biochem.* **55,** 106-119

75. Rankin, S. and Rozengurt, E. (1994) *J.Biol.Chem.* **269,** 704-710

76. Abedi, H., Dawes, K. E., and Zachary, I. (1995) *J.Biol.Chem.* **270,** 11367-11376

77. Matsumoto, K., Matsumoto, K., Nakamura, T., and Kramer, R. H. (1994) *J.Biol.Chem.* **269,** 31807-31813

78. Kharbanda, S., Saleem, A., Yuan, Z., Emoto, Y., Prasad, K. V., and Kufe, D. (1995) *Proc.Natl.Acad.Sci.U.S.A* **92,** 6132-6136

79. Sinnett-Smith, J., Zachary, I., Valverde, A. M., and Rozengurt, E. (1993) *J.Biol.Chem.* **268,** 14261-14268

80. Rankin, S., Morii, N., Narumiya, S., and Rozengurt, E. (1994) *FEBS Lett.* **354,** 315-319

81. Arregui, C. O., Balsamo, J., and Lilien, J. (1998) *J.Cell Biol.* **143,** 861-873

82. Liu, F., Sells, M. A., and Chernoff, J. (1998) *Mol.Cell Biol.* **18,** 250-259

83. Liu, M., Qin, Y., Liu, J., Tanswell, A. K., and Post, M. (1996) *J.Biol.Chem.* **271,** 7066-7071

84. Garton, A. J., Flint, A. J., and Tonks, N. K. (1996) *Mol.Cell Biol.* **16,** 6408-6418

85. Garton, A. J., Burnham, M. R., Bouton, A. H., and Tonks, N. K. (1997) *Oncogene* **15,** 877-885

86. Shen, Y., Schneider, G., Cloutier, J. F., Veillette, A., and Schaller, M. D. (1998) *J.Biol.Chem.* **273,** 6474-6481

87. Garton, A. J. and Tonks, N. K. (1999) *J.Biol.Chem.* **274,** 3811-3818

88. Shen, Y., Lyons, P., Cooley, M., Davidson, D., Veillette, A., Salgia, R., Griffin, J. D., and Schaller, M. D. (2000) *J.Biol.Chem.* **275,** 1405-1413

89. Angers-Loustau, A., Cote, J. F., Charest, A., Dowbenko, D., Spencer, S., Lasky, L. A., and Tremblay, M. L. (1999) *J.Cell Biol.* **144,** 1019-1031

90. Yu, D. H., Qu, C. K., Henegariu, O., Lu, X., and Feng, G. S. (1998) *J.Biol.Chem.* **273,** 21125-21131

91. Oh, E. S., Gu, H., Saxton, T. M., Timms, J. F., Hausdorff, S., Frevert, E. U., Kahn, B. B., Pawson, T., Neel, B. G., and Thomas, S. M. (1999) *Mol.Cell Biol.* **19,** 3205-3215

92. Liliental, J., Moon, S. Y., Lesche, R., Mamillapalli, R., Li, D., Zheng, Y., Sun, H., and Wu, H. (2000) *Curr.Biol.* **10,** 401-404

93. Fincham, V. J., Wyke, J. A., and Frame, M. C. (1995) *Oncogene* **10,** 2247-2252

94. Crouch, D. H., Fincham, V. J., and Frame, M. C. (1996) *Oncogene* **12,** 2689-2696

95. Wen, L. P., Fahrni, J. A., Troie, S., Guan, J. L., Orth, K., and Rosen, G. D. (1997) *J.Biol.Chem.* **272,** 26056-26061

96. Gervais, F. G., Thornberry, N. A., Ruffolo, S. C., Nicholson, D. W., and Roy, S. (1998) *J.Biol.Chem.* **273,** 17102-17108

97. Xu, L. H., Yang, X., Craven, R. J., and Cance, W. G. (1998) *Cell Growth Differ.* **9,** 999-1005

98. Cooray, P., Yuan, Y., Schoenwaelder, S. M., Mitchell, C. A., Salem, H. H., and Jackson, S. P. (1996) *Biochem.J.* **318 (Pt 1),** 41-47

99. Xing, Z., Chen, H. C., Nowlen, J. K., Taylor, S. J., Shalloway, D., and Guan, J. L. (1994) *Mol.Biol.Cell* **5,** 413-421

100. Sicheri, F., Moarefi, I., and Kuriyan, J. (1997) *Nature* **385,** 602-609

101. Xu, W., Harrison, S. C., and Eck, M. J. (1997) *Nature* **385,** 595-602

102. Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, S. L. (1994) *Cell* **76,** 933-945

103. Feng, S., Chen, J. K., Yu, H., Simon, J. A., and Schreiber, S. L. (1994) *Science* **266,** 1241-1247

104. Musacchio, A., Saraste, M., and Wilmanns, M. (1994) *Nat.Struct.Biol.* **1,** 546-551

105. Kmiecik, T. E. and Shalloway, D. (1987) *Cell* **49,** 65-73

106. Piwnica-Worms, H., Saunders, K. B., Roberts, T. M., Smith, A. E., and Cheng, S. H. (1987) *Cell* **49,** 75-82

107. Nada, S., Okada, M., MacAuley, A., Cooper, J. A., and Nakagawa, H. (1991) *Nature* **351,** 69-72

108. Liu, X., Brodeur, S. R., Gish, G., Songyang, Z., Cantley, L. C., Laudano, A. P., and Pawson, T. (1993) *Oncogene* **8,** 1119-1126

109. Alonso, G., Koegl, M., Mazurenko, N., and Courtneidge, S. A. (1995) *J.Biol.Chem.* **270,** 9840-9848

110. Alexandropoulos, K. and Baltimore, D. (1996) *Genes Dev.* **10,** 1341-1355

111. Kaplan, K. B., Swedlow, J. R., Morgan, D. O., and Varmus, H. E. (1995) *Genes Dev.* **9,** 1505-1517

112. Rohrschneider, L. R. (1980) *Proc.Natl.Acad.Sci.U.S.A* **77,** 3514-3518

113. Kaplan, K. B., Bibbins, K. B., Swedlow, J. R., Arnaud, M., Morgan, D. O., and Varmus, H. E. (1994) *EMBO J.* **13,** 4745-4756

114. Howell, B. W. and Cooper, J. A. (1994) *Mol.Cell Biol.* **14,** 5402-5411

115. Schaller, M. D., Hildebrand, J. D., and Parsons, J. T. (1999) *Mol.Biol.Cell* **10,** 3489-3505

116. Guinebault, C., Payrastre, B., Racaud-Sultan, C., Mazarguil, H., Breton, M., Mauco, G., Plantavid, M., and Chap, H. (1995) *J.Cell Biol.* **129,** 831-842

117. Guinebault, C., Payrastre, B., Mauco, G., Breton, M., Plantavid, M., and Chap, H. (1994) *Cell Mol.Biol.(Noisy.-le-grand)* **40,** 687-693

118. Skolnik, E. Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A., Ullrich, A., and Schlessinger, J. (1991) *Cell* **65,** 83-90

119. Bachelot, C., Rameh, L., Parsons, T., and Cantley, L. C. (1996) *Biochim.Biophys.Acta* **1311,** 45-52

120. Chen, H. C., Appeddu, P. A., Isoda, H., and Guan, J. L. (1996) *J.Biol.Chem.* **271,** 26329-26334

121. Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y., and Hirai, H. (1994) *EMBO J.* **13,** 3748-3756

122. Petch, L. A., Bockholt, S. M., Bouton, A., Parsons, J. T., and Burridge, K. (1995) *J.Cell Sci.* **108 (Pt 4),** 1371-1379

123. Nojima, Y., Morino, N., Mimura, T., Hamasaki, K., Furuya, H., Sakai, R., Sato, T., Tachibana, K., Morimoto, C., and Yazaki, Y. (1995) *J.Biol.Chem.* **270,** 15398-15402

124. Vuori, K. and Ruoslahti, E. (1995) *J.Biol.Chem.* **270,** 22259-22262

125. Vuori, K., Hirai, H., Aizawa, S., and Ruoslahti, E. (1996) *Mol.Cell Biol.* **16,** 2606-2613

126. Tachibana, K., Urano, T., Fujita, H., Ohashi, Y., Kamiguchi, K., Iwata, S., Hirai, H., and Morimoto, C. (1997) *J.Biol.Chem.* **272,** 29083-29090

127. Klemke, R. L., Leng, J., Molander, R., Brooks, P. C., Vuori, K., and Cheresh, D. A. (1998) *J.Cell Biol.* **140,** 961-972

128. Honda, H., Oda, H., Nakamoto, T., Honda, Z., Sakai, R., Suzuki, T., Saito, T., Nakamura, K., Nakao, K., Ishikawa, T., Katsuki, M., Yazaki, Y., and Hirai, H. (1998) *Nat.Genet.* **19,** 361-365

129. Matsuda, M., Mayer, B. J., Fukui, Y., and Hanafusa, H. (1990) *Science* **248,** 1537-1539

130. Turner, C. E., Glenney, J. R., Jr., and Burridge, K. (1990) *J.Cell Biol.* **111,** 1059-1068

131. Glenney, J. R., Jr. and Zokas, L. (1989) *J.Cell Biol.* **108,** 2401-2408

132. Salgia, R., Demetri, G. D., and Kaplan, W. D. (1994) *Cancer* **74,** 1887-1890

133. Brown, M. C., Perrotta, J. A., and Turner, C. E. (1996) *J.Cell Biol.* **135,** 1109-1123

134. Tachibana, K., Sato, T., D'Avirro, N., and Morimoto, C. (1995) *J.Exp.Med.* **182,** 1089-1099

135. Turner, C. E., Schaller, M. D., and Parsons, J. T. (1993) *J.Cell Sci.* **105 (Pt 3),** 637-645

136. Bellis, S. L., Miller, J. T., and Turner, C. E. (1995) *J.Biol.Chem.* **270,** 17437-17441

137. Cary, L. A., Chang, J. F., and Guan, J. L. (1996) *J.Cell Sci.* **109 (Pt 7),** 1787-1794

138. Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T., and Zipursky, S. L. (1996) *Cell* **85,** 639-650

139. Kundra, V., Escobedo, J. A., Kazlauskas, A., Kim, H. K., Rhee, S. G., Williams, L. T., and Zetter, B. R. (1994) *Nature* **367,** 474-476

140. Wennstrom, S., Siegbahn, A., Yokote, K., Arvidsson, A. K., Heldin, C. H., Mori, S., and Claesson-Welsh, L. (1994) *Oncogene* **9,** 651-660

141. Reiske, H. R., Kao, S. C., Cary, L. A., Guan, J. L., Lai, J. F., and Chen, H. C. (1999) *J.Biol.Chem.* **274,** 12361-12366

142. Petit, V., Boyer, B., Lentz, D., Turner, C. E., Thiery, J. P., and Valles, A. M. (2000) *J.Cell Biol.* **148,** 957-970

143. Yano, H., Uchida, H., Iwasaki, T., Mukai, M., Akedo, H., Nakamura, K., Hashimoto, S., and Sabe, H. (2000) *Proc.Natl.Acad.Sci.U.S.A* **97,** 9076-9081

144. Meredith, J. E., Jr., Fazeli, B., and Schwartz, M. A. (1993) *Mol.Biol.Cell* **4,** 953-961

145. Frisch, S. M. and Francis, H. (1994) *J.Cell Biol.* **124,** 619-626

146. Re, F., Zanetti, A., Sironi, M., Polentarutti, N., Lanfrancone, L., Dejana, E., and Colotta, F. (1994) *J.Cell Biol.* **127,** 537-546

147. Ruoslahti, E. and Reed, J. C. (1994) *Cell* **77,** 477-478

148. Boudreau, N., Sympson, C. J., Werb, Z., and Bissell, M. J. (1995) *Science* **267,** 891-893

149. Ilic, D., Genbacev, O., Jin, F., Caceres, E., Almeida, E. A., Bellingard-Dubouchaud, V., Schaefer, E. M., Damsky, C. H., and Fisher, S. J. (2001) *Am.J.Pathol.* **159,** 93-108

150. Zhu, X., Ohtsubo, M., Bohmer, R. M., Roberts, J. M., and Assoian, R. K. (1996) *J.Cell Biol.* **133,** 391-403

151. Bolen, J. B., Veillette, A., Schwartz, A. M., DeSeau, V., and Rosen, N. (1987) *Oncogene Res.* **1,** 149-168

152. Cartwright, C. A., Meisler, A. I., and Eckhart, W. (1990) *Proc.Natl.Acad.Sci.U.S.A* **87,** 558-562

153. Takekura, N., Yasui, W., Yoshida, K., Tsujino, T., Nakayama, H., Kameda, T., Yokozaki, H., Nishimura, Y., Ito, H., and Tahara, E. (1990) *Int.J.Cancer* **45,** 847-851

154. Fanning, P., Bulovas, K., Saini, K. S., Libertino, J. A., Joyce, A. D., and Summerhayes, I. C. (1992) *Cancer Res.* **52,** 1457-1462

155. Ottenhoff-Kalff, A. E., Rijksen, G., van Beurden, E. A., Hennipman, A., Michels, A. A., and Staal, G. E. (1992) *Cancer Res.* **52,** 4773-4778

156. Talamonti, M. S., Roh, M. S., Curley, S. A., and Gallick, G. E. (1993) *J.Clin.Invest* **91,** 53-60

157. Cartwright, C. A., Coad, C. A., and Egbert, B. M. (1994) *J.Clin.Invest* **93,** 509-515

158. Albelda, S. M. (1993) *Lab Invest* **68,** 4-17

159. Juliano, R. L. and Varner, J. A. (1993) *Curr.Opin.Cell Biol.* **5,** 812-818

160. Ruoslahti, E. (1999) *Adv.Cancer Res.* **76,** 1-20

161. Mizejewski, G. J. (1999) *Proc.Soc.Exp.Biol.Med.* **222,** 124-138

162. Sanders, R. J., Mainiero, F., and Giancotti, F. G. (1998) *Cancer Invest* **16,** 329-344

163. Weiner, T. M., Liu, E. T., Craven, R. J., and Cance, W. G. (1994) *Ann.Surg.Oncol.* **1,** 18-27

164. Owens, L. V., Xu, L., Craven, R. J., Dent, G. A., Weiner, T. M., Kornberg, L., Liu, E. T., and Cance, W. G. (1995) *Cancer Res.* **55,** 2752-2755

165. Cance, W. G., Harris, J. E., Iacocca, M. V., Roche, E., Yang, X., Chang, J., Simkins, S., and Xu, L. (2000) *Clin.Cancer Res.* **6,** 2417-2423

166. Su, J. M., Gui, L., Zhou, Y. P., and Zha, X. L. (2002) *World J.Gastroenterol.* **8,** 613-618

167. Han, N. M., Fleming, R. Y., Curley, S. A., and Gallick, G. E. (1997) *Ann.Surg.Oncol.* **4,** 264-268

168. Owens, L. V., Xu, L., Dent, G. A., Yang, X., Sturge, G. C., Craven, R. J., and Cance, W. G. (1996) *Ann.Surg.Oncol.* **3,** 100-105

169. Tremblay, L., Hauck, W., Aprikian, A. G., Begin, L. R., Chapdelaine, A., and Chevalier, S. (1996) *Int.J.Cancer* **68,** 164-171

170. Rovin, J. D., Frierson, H. F., Jr., Ledinh, W., Parsons, J. T., and Adams, R. B. (2002) *Prostate* **53,** 124-132

171. Kornberg, L. J. (1998) *Head Neck* **20,** 745-752

172. Judson, P. L., He, X., Cance, W. G., and Van Le, L. (1999) *Cancer* **86,** 1551-1556

173. Zagzag, D., Friedlander, D. R., Margolis, B., Grumet, M., Semenza, G. L., Zhong, H., Simons, J. W., Holash, J., Wiegand, S. J., and Yancopoulos, G. D. (2000) *Pediatr.Neurosurg.* **33,** 49-55

174. Wang, D., Grammer, J. R., Cobbs, C. S., Stewart, J. E., Jr., Liu, Z., Rhoden, R., Hecker, T. P., Ding, Q., and Gladson, C. L. (2000) *J.Cell Sci.* **113 Pt 23,** 4221-4230

175. Jones, G., Machado, J., Jr., Tolnay, M., and Merlo, A. (2001) *Cancer Res.* **61,** 5688-5691

176. Hecker, T. P., Grammer, J. R., Gillespie, G. Y., Stewart, J., Jr., and Gladson, C. L. (2002) *Cancer Res.* **62,** 2699-2707

177. Chan, P. Y., Kanner, S. B., Whitney, G., and Aruffo, A. (1994) *J.Biol.Chem.* **269,** 20567-20574

178. Schlaepfer, D. D., Hauck, C. R., and Sieg, D. J. (1999) *Prog.Biophys.Mol.Biol.* **71,** 435-478

179. Maung, K., Easty, D. J., Hill, S. P., and Bennett, D. C. (1999) *Oncogene* **18,** 6824-6828

180. Xu, L. H., Owens, L. V., Sturge, G. C., Yang, X., Liu, E. T., Craven, R. J., and Cance, W. G. (1996) *Cell Growth Differ.* **7,** 413-418

181. Xu, L. H., Yang, X., Bradham, C. A., Brenner, D. A., Baldwin, A. S., Jr., Craven, R. J., and Cance, W. G. (2000) *J.Biol.Chem.*

182. Carragher, N. O., Fincham, V. J., Riley, D., and Frame, M. C. (2001) *J.Biol.Chem.* **276,** 4270-4275

183. Carragher, N. O., Westhoff, M. A., Riley, D., Potter, D. A., Dutt, P., Elce, J. S., Greer, P. A., and Frame, M. C. (2002) *Mol.Cell Biol.* **22,** 257-269

184. Chan, P. C., Liang, C. C., Yu, K. C., Chang, M. C., Ho, W. L., Chen, B. H., and Chen, H. C. (2002) *J.Biol.Chem.*

185. Renshaw, M. W., Price, L. S., and Schwartz, M. A. (1999) *J.Cell Biol.* **147,** 611-618

186. Leyton, J., Garcia-Marin, L. J., Tapia, J. A., Jensen, R. T., and Moody, T. W. (2001) *Cancer Lett.* **162,** 87-95

187. Slack, J. K., Adams, R. B., Rovin, J. D., Bissonette, E. A., Stoker, C. E., and Parsons, J. T. (2001) *Oncogene* **20,** 1152-1163

188. Zheng, D. Q., Woodard, A. S., Fornaro, M., Tallini, G., and Languino, L. R. (1999) *Cancer Res.* **59,** 1655-1664

189. Hauck, C. R., Sieg, D. J., Hsia, D. A., Loftus, J. C., Gaarde, W. A., Monia, B. P., and Schlaepfer, D. D. (2001) *Cancer Res.* **61,** 7079-7090

190. Hemler, M. E. (2001) *J.Cell Biol.* **155,** 1103-1107

191. Kohno, M., Hasegawa, H., Miyake, M., Yamamoto, T., and Fujita, S. (2002) *Int.J.Cancer* **97,** 336-343

192. Li, X., Regezi, J., Ross, F. P., Blystone, S., Ilic, D., Leong, S. P., and Ramos, D. M. (2001) *J.Cell Sci.* **114,** 2665-2672

193. Jones, G., Machado, J., Jr., and Merlo, A. (2001) *Cancer Res.* **61,** 4978-4981

194. Legrand, C., Gilles, C., Zahm, J. M., Polette, M., Buisson, A. C., Kaplan, H., Birembaut, P., and Tournier, J. M. (1999) *J.Cell Biol.* **146,** 517-529

195. Shibata, K., Kikkawa, F., Nawa, A., Thant, A. A., Naruse, K., Mizutani, S., and Hamaguchi, M. (1998) *Cancer Res.* **58,** 900-903

196. Sein, T. T., Thant, A. A., Hiraiwa, Y., Amin, A. R., Sohara, Y., Liu, Y., Matsuda, S., Yamamoto, T., and Hamaguchi, M. (2000) *Oncogene* **19,** 5539-5542

197. Iwata, S., Ohashi, Y., Kamiguchi, K., and Morimoto, C. (2000) *J.Dermatol.Sci.* **23,** 75-86

198. McLean, G. W., Brown, K., Arbuckle, M. I., Wyke, A. W., Pikkarainen, T., Ruoslahti, E., and Frame, M. C. (2001) *Cancer Res.* **61,** 8385-8389

199. Liu, D., Aguirre, G. J., Estrada, Y., and Ossowski, L. (2002) *Cancer Cell* **1,** 445-457

200. Yamamura, S., Hakomori, S., Wada, A., and Igarashi, Y. (2000) *Ann.N.Y.Acad.Sci.* **905,** 301-307

201. Nurcombe, V., Smart, C. E., Chipperfield, H., Cool, S. M., Boilly, B., and Hondermarck, H. (2000) *J.Biol.Chem.*

202. Lin, V. C., Ng, E. H., Aw, S. E., Tan, M. G., Ng, E. H., and Bay, B. H. (2000) *Mol.Endocrinol.* **14,** 348-358

203. Schwartz, M. A. and Ginsberg, M. H. (2002) *Nat.Cell Biol.* **4,** E65-E68

204. Ignatoski, K. M. and Ethier, S. P. (1999) *Breast Cancer Res.Treat.* **54,** 173-182

205. Ignatoski, K. M., Maehama, T., Markwart, S. M., Dixon, J. E., Livant, D. L., and Ethier, S. P. (2000) *Br.J.Cancer* **82,** 666-674

206. Brunton, V. G., Ozanne, B. W., Paraskeva, C., and Frame, M. C. (1997) *Oncogene* **14,** 283-293

207. Lu, Z., Jiang, G., Blume-Jensen, P., and Hunter, T. (2001) *Mol.Cell Biol.* **21,** 4016-4031

208. Beviglia, L., Matsumoto, K., Lin, C. S., Ziober, B. L., and Kramer, R. H. (1997) *Int.J.Cancer* **74,** 301-309

209. Ferracini, R., Di Renzo, M. F., Scotlandi, K., Baldini, N., Olivero, M., Lollini, P., Cremona, O., Campanacci, M., and Comoglio, P. M. (1995) *Oncogene* **10,** 739-749

210. Di Renzo, M. F., Poulsom, R., Olivero, M., Comoglio, P. M., and Lemoine, N. R. (1995) *Cancer Res.* **55,** 1129-1138

211. Di Renzo, M. F., Olivero, M., Giacomini, A., Porte, H., Chastre, E., Mirossay, L., Nordlinger, B., Bretti, S., Bottardi, S., Giordano, S., and . (1995) *Clin.Cancer Res.* **1,** 147-154

212. Giordano, S., Bardelli, A., Zhen, Z., Menard, S., Ponzetto, C., and Comoglio, P. M. (1997) *Proc.Natl.Acad.Sci.U.S.A* **94,** 13868-13872

213. Chen, H. C., Chan, P. C., Tang, M. J., Cheng, C. H., and Chang, T. J. (1998) *J.Biol.Chem.* **273,** 25777-25782

214. Beviglia, L. and Kramer, R. H. (1999) *Int.J.Cancer* **83,** 640-649

215. Maulik, G., Kijima, T., Ma, P. C., Ghosh, S. K., Lin, J., Shapiro, G. I., Schaefer, E., Tibaldi, E., Johnson, B. E., and Salgia, R. (2002) *Clin.Cancer Res.* **8,** 620-627

216. Rahimi, N., Tremblay, E., McAdam, L., Park, M., Schwall, R., and Elliott, B. (1996) *Cell Growth Differ.* **7,** 263-270

217. Nakaigawa, N., Weirich, G., Schmidt, L., and Zbar, B. (2000) *Oncogene* **19,** 2996-3002

218. Igishi, T., Fukuhara, S., Patel, V., Katz, B. Z., Yamada, K. M., and Gutkind, J. S. (1999) *J.Biol.Chem.* **274,** 30738-30746

219. Shu, H. K., Pelley, R. J., and Kung, H. J. (1990) *Proc.Natl.Acad.Sci.U.S.A* **87,** 9103-9107

220. Hofstra, R. M., Landsvater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J. W., van Amstel, H. K., and Romeo, G. (1994) *Nature* **367,** 375-376

221. Tavormina, P. L., Rimoin, D. L., Cohn, D. H., Zhu, Y. Z., Shiang, R., and Wasmuth, J. J. (1995) *Hum.Mol.Genet.* **4,** 2175-2177

222. Webster, M. K., D'Avis, P. Y., Robertson, S. C., and Donoghue, D. J. (1996) *Mol.Cell Biol.* **16,** 4081-4087

223. Reynolds, A. B., Roesel, D. J., Kanner, S. B., and Parsons, J. T. (1989) *Mol.Cell Biol.* **9,** 629-638

224. Young, J. A., Bates, P., and Varmus, H. E. (1993) *J.Virol.* **67,** 1811-1816

225. Thomas, F., Wu, J., Contreras, J. L., Smyth, C., Bilbao, G., He, J., and Thomas, J. (2001) *Surgery* **130,** 333-338

226. Laemmli, U. K. (1970) *Nature* **227,** 680-685

227. Smith, D. B. and Johnson, K. S. (1988) *Gene* **67,** 31-40

228. Keely, P. J., Fong, A. M., Zutter, M. M., and Santoro, S. A. (1995) *J.Cell Sci.* **108 (Pt 2),** 595-607

229. Schaller, M. D. and Parsons, J. T. (1995) *Mol.Cell Biol.* **15,** 2635-2645

230. Johnson, L. N., Noble, M. E., and Owen, D. J. (1996) *Cell* **85,** 149-158

231. Schlaepfer, D. D. and Hunter, T. (1997) *J.Biol.Chem.* **272,** 13189-13195

232. Thomas, S. M., Soriano, P., and Imamoto, A. (1995) *Nature* **376,** 267-271

233. Schlaepfer, D. D., Jones, K. C., and Hunter, T. (1998) *Mol.Cell Biol.* **18,** 2571-2585

234. Parsons, J. T., Martin, K. H., Slack, J. K., Taylor, J. M., and Weed, S. A. (2000) *Oncogene* **19,** 5606-5613

235. Zhang, X., Chattopadhyay, A., Ji, Q. S., Owen, J. D., Ruest, P. J., Carpenter, G., and Hanks, S. K. (1999) *Proc.Natl.Acad.Sci.U.S.A* **96,** 9021-9026

236. Han, D. C. and Guan, J. L. (1999) *J.Biol.Chem.* **274,** 24425-24430

237. Taylor, J. M., Hildebrand, J. D., Mack, C. P., Cox, M. E., and Parsons, J. T. (1998) *J.Biol.Chem.* **273,** 8063-8070

238. Taylor, J. M., Macklem, M. M., and Parsons, J. T. (1999) *J.Cell Sci.* **112 (Pt 2),** 231-242

239. Zhao, J., Zheng, C., and Guan, J. (2000) *J.Cell Sci.* **113,** 3063-3072

240. Chan, P. C., Lai, J. F., Cheng, C. H., Tang, M. J., Chiu, C. C., and Chen, H. C. (1999) *J.Biol.Chem.* **274,** 26901-26906

241. Sonoda, Y., Matsumoto, Y., Funakoshi, M., Yamamoto, D., Hanks, S. K., and Kasahara, T. (2000) *J.Biol.Chem.* **275,** 16309-16315

242. Willke, A. and Tural, D. (1987) *Mikrobiyol.Bul.* **21,** 279-283

243. Tang, H., Kerins, D. M., Hao, Q., Inagami, T., and Vaughan, D. E. (1998) *J.Biol.Chem.* **273,** 18268-18272

244. Tang, C. K., Concepcion, X. Z., Milan, M., Gong, X., Montgomery, E., and Lippman, M. E. (1999) *Cancer Res.* **59,** 5315-5322

245. van Golen, K. L., Risin, S., Staroselsky, A., Berger, D., Tainsky, M. A., Pathak, S., and Price, J. E. (1996) *Clin.Exp.Metastasis* **14,** 95-106

246. van Golen, K. L., Wu, Z. F., Qiao, X. T., Bao, L. W., and Merajver, S. D. (2000) *Cancer Res.* **60,** 5832-5838

247. Wang, B., Soule, H. D., and Miller, F. R. (1997) *Anticancer Res.* **17,** 4387-4394

248. Hanigan, M. H., Gallagher, B. C., Townsend, D. M., and Gabarra, V. (1999) *Carcinogenesis* **20,** 553-559

249. Keydar, I., Chen, L., Karby, S., Weiss, F. R., Delarea, J., Radu, M., Chaitcik, S., and Brenner, H. J. (1979) *Eur.J.Cancer* **15,** 659-670

250. Zrihan-Licht, S., Fu, Y., Settleman, J., Schinkmann, K., Shaw, L., Keydar, I., Avraham, S., and Avraham, H. (2000) *Oncogene* **19,** 1318-1328

251. Cooley, M. A., Broome, J. M., Ohngemach, C., Romer, L. H., and Schaller, M. D. (2000) *Mol.Biol.Cell* **11,** 3247-3263

252. Cukierman, E., Pankov, R., Stevens, D. R., and Yamada, K. M. (2001) *Science* **294,** 1708-1712

253. Cukierman, E., Pankov, R., and Yamada, K. M. (2002) *Curr.Opin.Cell Biol.* **14,** 633-639

254. Park, W. S., Dong, S. M., Kim, S. Y., Na, E. Y., Shin, M. S., Pi, J. H., Kim, B. J., Bae, J. H., Hong, Y. K., Lee, K. S., Lee, S. H., Yoo, N. J., Jang, J. J., Pack, S., Zhuang, Z., Schmidt, L., Zbar, B., and Lee, J. Y. (1999) *Cancer Res.* **59,** 307-310

255. Santoro, M. M., Penengo, L., Minetto, M., Orecchia, S., Cilli, M., and Gaudino, G. (1998) *Oncogene* **17,** 741-749

256. Nagata, H., Worobec, A. S., Oh, C. K., Chowdhury, B. A., Tannenbaum, S., Suzuki, Y., and Metcalfe, D. D. (1995) *Proc.Natl.Acad.Sci.U.S.A* **92,** 10560-10564

APPENDIX F

Era of Hope. Department of Defense Breast Cancer Research Program Meeting.

Orange County Convention Center

Orlando, Florida

September 25-28, 2002

A CELL MODEL SYSTEM TO STUDY THE ROLE OF FOCAL ADHESION KINASE IN THE ACQUISITION OF CANCER PHENOTYPES

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Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that receives and transmits signals from the extracellular environment. Upon cell adhesion, extracellular matrix components bind to their cell surface receptors, the integrins, causing them to cluster. Integrin clustering, leads to FAK activation. FAK is known to mediate a variety of normal cellular processes including cell motility, cell survival, and cell cycle regulation. Thus, aberration of FAK signals could lead to critical pathological effects, including cancer. Interestingly, FAK has been found to be overexpressed in a variety of tumors and cancer cells. Therefore, FAK is likely to play a role in oncogenesis. In this study, cell model systems have been established to study the role of FAK signaling in the acquisition and progression of cancer phenotypes. In the first model system, the normal immortalized epithelial cell line, MCF10A, has been modified to enhance FAK signaling. In order to elevate FAK signaling, wild type FAK and an activated mutant of FAK, SuperFAK, were expressed in MCF10A. SuperFAK contains a double (K to E) mutation in the activation loop of the kinase domain which increases its kinase activity. In turn, SuperFAK can phosphorylate FAK substrates to higher levels than wild type FAK. In the second model system, FAK was inhibited in a breast cancer epithelial cell line, T47D, using FRNK, a naturally occurring spliced variant of FAK that acts as a dominant negative of FAK. Upon achieving changes in FAK signaling, the MCF10A and T47D cells were monitored for the acquisition or loss of transformation phenotypes. The elevation of FAK signaling alone had no effect in the adhesion independent growth of the MCF10A cells. Interestingly, the addition of high levels of EGF to FAK and SuperFAK expressing MCF10As was sufficient allow them to form colonies in a soft agar system. Conversely, when FRNK was expressed in the T47D cancer cells a dramatic loss in their adhesion independent growth was observed. The underlying mechanisms for some of the observed effects are being investigated. Furthermore, the MCF10A and T47D cells in which FAK signaling has been successfully altered are being tested for further phenotypic changes *in vitro* and *in vivo*. The data collected indicates the potential importance of aberrant FAK signaling as a cause for some of the phenotypic changes that occur when a cell becomes oncogenically transformed.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0377 supported this work.

APPENDIX G

DEGREES OBTAINED.

Veronica Gabarra-Niecko successfully defended her Ph.D. on April 1, 2003.

THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL

The Graduate School

DOCTORAL EXAM REPORT FORM

(NOTE: The Committee Composition form should be on file with the Graduate School **before** exam results are reported.)

Student's Name Veronica Gabarra Niecko PID# 703734086

Department/Curriculum/School: Cell Biology & Anatomy/School of Medicine

PART I: REPORT OF PRELIMINARY WRITTEN EXAMINATION

On behalf of a majority of the examining committee, I certify that the above named student:

✓ successfully passed the examination
____ failed to pass the examination
check here if student previously failed exam. Date(s): _____

W. Joseph Costello — *signature of committee chair*
7/11/97 — *date*

PART II: REPORT OF ORAL EXAMINATION

On behalf of a majority of the examining committee, I certify that the above named student:

✓ successfully passed the examination
____ failed to pass the examination
check here if student previously failed exam. Date(s): _____

signature of committee chair
9/1/2000 — *date*

PART III: REPORT OF THE FINAL ORAL EXAMINATION (defense of dissertation)

A majority of the committee for the above named student has judged the dissertation and its defense to be:

✓ acceptable
____ unacceptable

signature of committee chair
April 1/02 — *date*

Committee member name/signature	Pass/Fail	Committee member name/signature	Pass/Fail
Mike Schaller	P		
Ellen R Weiss	P		
William Catterall	P		
Kathleen Burridge	P		
Rudy Juliano	P		

Instructions:
- submit page 4 to the Graduate School when Part I is complete
- submit page 3 to the Graduate School when Part II is complete
- submit page 1 to the Graduate School when Part III is complete
- keep page 2 for your files